

No relief from poverty's debt

This year's annual jamboree of the International Monetary Fund and the World Bank in Washington two weeks ago has deservedly earned a bad press. Their neglect of poor countries' debts is their most serious failing.

BOTH the International Monetary Fund (IMF) and the World Bank, like the United Nations Organization, are products of that brief spell towards the end of the Second World War when there was an almost general belief that the creation of enlightened institutions would itself spread enlightenment. Both organizations, created along with the Bretton Woods package that gave the world currency stability of a kind between 1945 and the early 1970s, are banks of a kind — and, like more ordinary banks, they ask a price for the help they give. One, the IMF, exists to help members over temporary difficulties, uncomfortably large gaps between imports and exports, for example. The usual price is that assisted governments must follow economic policies dictated by IMF, which they usually find unpalatable. The other, the World Bank, is more like a merchant bank, designed to invest rich countries' capital in poor countries with the hope that they will become less so; the interest rates it charges are low, but rich as well as poor countries are calculated eventually to benefit from the expansion of world trade.

This year's particular complaint is that the two banks, but especially the IMF, have been exuding complacency when the problems confronting them are horrendous. The problems, of course, are those of the poor countries of the world and their mountains of debt — \$300,000 million in round numbers. It seems generally to be agreed, even among the commercial banks that are the poor countries' chief creditors, that much of this debt will never be repaid. Why else would the commercial banks feel bound to write down the value of the poor-country debts they carry on their books? But it is one thing for the commercial banks to admit to themselves and their shareholders that their assets are worth less than they had believed, and quite a different thing for them to forgive a particular country's debt even when it is manifestly incapable of paying the interest on its loans, let alone repaying instalments of the capital. That, the banks say, would be a recipe for getting none of their money back. So the banks and the indebted countries are forced to play a kind of poker game whose objective is a redistribution and overall reduction of indebtedness. An international solution, of a kind that the IMF and the World Bank are designed to devise, would be better.

But have there not already been plans for solving the poor countries' debt problems? As it happens, the present Secretary of the US Treasury, Mr Nicholas Brady, and his

immediate predecessor (now US Secretary of State), Mr James Baker, are at least the nominal authors of two successive plans to put the poor countries of the world back on reasonable tracks. The Baker plan was a suggestion that commercial banks should trade some of their outstanding debt for governmental bonds of discounted value or even for equity, perhaps in state or commercial enterprises. The difficulty proved to be that even if commercial banks would settle on such disadvantageous terms, they would then be unwilling to lend the further funds that the poor countries need. The Brady plan acknowledged that difficulty by supposing that the IMF and the World Bank would lubricate the transition from the present state of affairs to a more manageable future by using their funds to back arrangements between commercial banks and poor countries.

It is now apparent that even this plan will not work, not least because the members of the IMF and the World Bank have let their meeting pass without voting the extra capital the two banks would need to carry out the task urged on them by Brady. The reasons may have been procedural, but the outcome is inexcusable. But it seems also now to be plain that the reconstruction of Mexico's foreign indebtedness, hailed earlier this year as the first success of the Brady plan, may come unstuck, because the commercial banks that have written down their debts are unwilling to throw good money after bad, as they see it. If that happens, despite Mexico's willingness to follow IMF recipes for economic reform, it will fall to the United States to pull some awkward chestnuts from the fire.

Meanwhile, there is no prospect that piecemeal solutions of individual debtors' problems will put the world to rights. The need is for a wholesale solution. How to achieve that? Hard though it may seem, it is right and proper that countries that have hopelessly mismanaged their affairs should not be relieved of their debt — but the prospect that there might be a global settlement could be a more powerful incentive to reform than the individual hectorings of creditor banks are now. But even among the governments doing their level best, there is a danger that any global settlement will bear most harshly on those that have tried hardest, and which might be judged best able to repay in full, or something like it. But there are ways round that. Western commercial banks are forgivably shy of ending up with equity holdings in distant impoverished places, but would the same be true



of well-intentioned debtor governments for example? That is the sort of stratagem that the IMF should be exploring. If it cannot, there may be no choice but an international conference of debtors and creditors at which they hammer out a settlement in concert. The present mess cannot be allowed to continue. □

Amis and MacGregor

British universities cannot expand a second time without qualitative change.

WHEN the first great expansion of British higher education was set in train by the Robbins Report in 1963, public argument in Britain was quickly polarized around Mr Kingsley Amis's complaint that "more means worse". Mr John MacGregor, the British Secretary of State for Education and Science, has evoked none of that trouble by his casual announcement that his government now hopes that the participation rate in higher education (the percentage of school leavers following degree courses either then or later) will increase to 23 per cent by the end of the century (see *Nature* **341**, 371; 1989). But MacGregor will not be able to forget Amis's complaint for long. Already, the vice-chancellors (otherwise rectors or presidents, according to geography) of British universities have made it plain that there will be a reduction of the quality of higher education if the government does not meet the cost of teaching extra students. That is inescapable, but only half the problem.

The error — some would say the folly — of the then government's response to the Robbins report was its assumption that a twofold expansion could be accomplished without a qualitative change in teaching methods. One result was the creation of a new crop of universities in the image of pre-existing universities. Only now have the authorities concluded that many British universities are too small to sustain substantial research programmes — or even to be full universities, teaching philosophy as well as physics. (Some have dropped both.) Both the universities and the British government should be careful to avoid that mistake a second time.

The dilemma for both is worsened by the otherwise welcome prospect that secondary education will in future be less specialized. Students at secondary schools will no longer be expected to win places in specialized courses at universities by virtually demonstrating that they can already do what may afterwards be required of them. But universities have traditionally complained that they cannot take young people through the traditional British specialized degree course in three years if students are a whit less well-prepared than at present. The best solution must surely be that some universities should become four-year universities. The question then is merely whether the government will meet the extra cost. On the face of things, it has no choice.

There are intellectual as well as administrative prob-

lems for the universities to tackle. The truth is that the extra 10 per cent of young people McGregor is urging towards higher education will have different needs. Britain's higher education is already notorious for production engineers who do not understand economics, historians who are computer-illiterate and scientists who know only English, and that sometimes imperfectly. What will the British system of higher education, jealous of its independence and traditions, do to accommodate the different needs of its new students? Especially now that 1992 is not far away? □

Benefactor needed

There needs to be an imaginative response to a Soviet offer to create an international centre on Lake Baikal.

NOT so long ago, it was common to hear complaints that the Soviet government was allowing the ruin of one of the world's most remarkable natural monuments, the huge inland sea against the border with Mongolia known as Lake Baikal. It is true, of course, that for a long time bureaucratic impediments exaggerated the natural difficulties of reaching this remote place, so that pollution of the world's largest natural reservoir of fresh water could not be independently monitored and assessed. The good news is that the alarms of the past few decades have been without foundation; Lake Baikal remains more or less in its pristine state. The remarkable news is that the Soviet authorities are deadly serious in their willingness to throw Lake Baikal open to international research (see page 481). Even if the rest of the world does not quickly seize the opportunity, there is already enough collaborative work under way to make a respectable research programme.

But it must be hoped that matters will not rest there. The plans put forward are for a research centre sited on the lake whose programme will be determined by researchers, and not by other interests. The objective is not simply to learn more about Lake Baikal, but to tell whether this distinctive body of water holds the key to other contemporary puzzles, the record of climatic change and the pace of tectonic or even evolutionary change, for example. Practical benefits, especially in the development of monitoring techniques, are an extra.

The difficulty, as always, is the accomplishment of this objective. There will be a need, in the next few years, for something like \$20 million in hard currency to establish the infrastructure of a working research centre. It would be preferable if governments were not directly involved, although they are the most obvious sources of funds on such a scale. (Russians, accustomed to print money when they need more of it, do not appreciate the struggles required in the West to raise sums of this kind, but Mr Gorbachev's promised budget reforms may change all that.) Could this be a case where one of the larger foundations will recognize that the time has come to back an imaginative scheme imaginatively? □

Australian innovation covered by US patent

- 'Gene shears' invented in Colorado in 1986
- Commercial deal with French may be empty

Sydney

CLAIMS that a genetic-engineering technique, popularly called 'gene shears', may turn out to be one of Australia's most profitable scientific discoveries are turning sour, for the elementary reason that a US company filed for patent rights on the same discovery in 1986. And the dispute over priorities raises serious questions about the true worth of a A\$22-million (£11 million) commercial development deal that the Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO) made with a French company, Groupe Limagrain.

The ability of ribozymes to cut RNA molecules at specific sequences was discovered by Thomas Cech, working at the University of Colorado in the early 1980s. He made a broad patent application in 1986, although it may well be two more years before a US patent is issued, and even longer in Europe. Cech's technique was developed further in 1987 by Olke Uhlenbeck, also at the University of Colorado, using 'hammerhead' ribozymes. It is not known whether Uhlenbeck has applied for a patent on his part of the work.

In August last year, Jim Haseloff and Wayne Gerlach from the Plant Industry Division of CSIRO published a paper in *Nature* (334, 585-591), describing a refined version of Uhlenbeck's hammerhead ribozyme technique, able to act on a greater range of RNA sequences, and with greater specificity, than had been shown before. And in July this year, to much fanfare, CSIRO announced a joint venture with the French agricultural concern Groupe Limagrain to find commercial applications for the gene-shears technology.

Making no mention of Cech or Uhlenbeck, CSIRO announced that it held worldwide patents to the discovery, and that Australia would benefit from the numerous potential applications of the technology in medicine and plant and animal science. But lawyers for the US Biochemical Corporation (USB), which represents Cech and has sole rights to the technology he developed, responded quickly to the Australian announcements.

USB is so confident of the pre-eminence of its claim that it intends to announce, on 20 October, the formation of a new corporate subdivision dedicated solely to the commercial development of ribozyme technology. Earlier this year, USB announced the first product, RNAzyme-

Tet.1.0 Ribozyme, based on the new technology, and senior vice-president Vincent Kazmer says that the product would not have been launched "unless USB was confident of being awarded the patent".

Richard Warburg of Fish and Richardson, lawyers for USB and the University of Colorado, supports Kazmer's assertion, saying that Cech's patent application is broad enough to cover any ribozyme, and that any patent granted to CSIRO would be dominated by Cech's.

It now seems that Jim Peacock, head of the Plant Industry Division at CSIRO, had been advised by the Australian Patent Office (APO) that Cech's and Uhlenbeck's papers constituted 'prior art' as early as April this year, before he made widely reported claims that came out in July. "We don't think they [CSIRO] will get their patent", said Stephen Castle of the APO, which has given Uhlenbeck's paper a 'category X' classification, meaning that it is of the greatest relevance with respect to CSIRO's claim.

Peacock says that he was aware of the Cech patent application and the Uhlenbeck work when CSIRO applied for its patent. But he says he cannot remember the APO analysis, although it is automatic for APO to provide its recommendations when a group applies for an international patent through its national office.

CSIRO is about to publish the first example of gene shears technology used *in vivo*, in the *Proceedings of the National Academy of Sciences of the USA*. Because of the extent of Cech's possible patent protection, it is unlikely, according to Warburg, that even in this area of *in vivo* work CSIRO will be able to practise its technology without a licence. "Cech's application made broad and narrow claims. If CSIRO is entitled to anything it is to a narrow claim, the practice of which will still fall within the broader claims made by Cech".

Tania Ewing

Paris

Groupe Limagrain's legal adviser said last week that a literature search had been carried out before the agreement with CSIRO was signed, but added that he was in the process of studying the dossier on gene shears. He would not say whether Groupe Limagrain had decided to go ahead with the investment with full knowledge of the US patent. Last Monday, a company official said that the company had "no further information about this patent at the moment".

Peter Coles

ANTARCTIC RESEARCH

Greenhouse seen from South Pole?

London

A SIGNIFICANT loss of snow and ice near the British Antarctic Survey (BAS) base at Rothera has been recorded, according to a Natural Environment Research Council (NERC) report, but whether the inferred temperature rise is symptomatic of greenhouse warming or the result of a natural climate cycle is uncertain.

The report, primarily designed to assess the need for a new airstrip at Rothera, concludes that the importance of research into global climate change outweighs the damage to the environment that an airstrip would entail.

Since 1982, there has been a dramatic rise of 1°C in the mean summer air temperature during January and February, and Dr Lewis Smith, of the BAS, says that evidence of a warming had been recorded at Rothera since 1950. Although the recent rise may seem small, Smith added, it is significant for the polar regions where the ice-cover is very sensitive to temperature changes. The rapid recession of the ice-



Snow-covered mountains in Antarctica.

cover has occurred more swiftly in the South Orkney Islands.

But greenhouse warming is not the only explanation: the world is experiencing a natural warming cycle which is thought to be at a peak. Smith is concerned the greenhouse effect may exacerbate the cycle or delay the expected downturn in temperature.

Dr John King, an Earth scientist at the BAS, said one has to be "very careful before leaping in" and talking about greenhouse warming. Models predicting the environmental effect of a doubling in carbon dioxide suggest that there would be no detectable change in temperature. However, King said simulations of future climates indicate that the first areas to suffer would be the marginal polar regions where the temperature of sea ice is "closely tied up" with land-ice conditions. Ben Webb

■ A meeting of the signatory nations of the Antarctic Treaty started in Paris this week. Britain will oppose the plan to declare Antarctic an international wilderness reserve while France and Australia will urge that the Antarctic Treaty be replaced with a declaration to protect the Antarctic environment. □

Break-up of monopoly?

Tokyo

JAPAN'S domestic telecommunications giant, Nippon Telegraph and Telephone (NTT), may be broken up into regional companies if recommendations in an interim report released last week by the Telecommunications Council, an advisory body to the Ministry of Posts and Telecommunications, are followed. But resistance to break-up is strong and it is not clear what path the government will take when the council's final recommendations are issued next March.

With 270,000 employees and an operating income of about \$45,000 million, NTT is one of the largest companies in the world. Although it was privatized in 1985, and three competitors entered the market, NTT still controls about 98 per cent of the market, and long-distance domestic telecommunications charges remain high. Japan's international telecommunications charges, in contrast, are tumbling as a result of competition (see below).

Of a number of ways to break up the NTT monopoly, the council favours a split into one long-distance company and 11 regional companies, similar to the break-up of the US monopoly American Telephone and Telegraph (AT&T).

The council argues that the split will

promote competition, reduce rates for users, and turn NTT into a more productive entity. But both the powerful Japan Telecommunications Workers Union, to which NTT employees belong, and NTT president Haruo Yamaguchi have come out publicly against the council's recommendations. Yamaguchi says that break-up would increase local rates and create regional differences in charges, and adds that it would interfere with NTT's development of a digital Information Network System (INS) and weaken NTT's research and development capabilities. But opinions in NTT management are said to be divided, with those on the sales and administrative side favouring restructuring while those with an engineering background tend to oppose it.

Proposals to split up NTT when it was

HYDROELECTRICITY

Indian dams go ahead

Bangalore

To the disappointment of Indian environmentalists, the World Bank has not only agreed to provide around 10 per cent of the estimated cost of the gigantic Narmada Sagar-Sardar Sarovar dam project but has also expressed its satisfaction over the plans drawn up to resettle the people evacuated to make room for it. The US Environmental Defense Fund and Environmental Policy Institute had earlier appealed to the World Bank to withdraw its support, but the bank has apparently been impressed by the work of Sanat Mehta, chairman of the Sardar-Sarovar Narmada Sagar Nigam, an agency charged with the task of speeding up the project and dealing with the evacuees.

In its present form, the Narmada Sagar and Sardar-Sarovar project together are designed to yield about 2,500 MW of hydroelectric power and will irrigate about 2 million hectares. The entire scheme involves the construction of 30 major dams, and many smaller ones, on the Narmada river and its tributaries. Mehta says that those affected will be given irrigable land, housing plots, agricultural aid, resettlement grants, free transport and civic amenities. This offer is, he says, "unparalleled and unmatched in the world". But a recent study commissioned by the Indian National Trust for Art and Cultural Heritage (INTACH) concludes that the objectives set out by the planners will not be achieved with the present plans. V. Paranjyoti of INTACH fears that the whole project will come to a halt within two or three years for lack of funds, and that money intended for rehabilitation will go elsewhere.

Radhakrishna Rao

privatized in 1985 were shelved because of strong opposition from NTT and some members of the ruling Liberal Democratic Party. Now, however, NTT has lost a lot of political influence because of its involvement in the Recruit scandal which forced the resignation of the then Prime Minister. But NTT still has many powerful friends, including the Federation of Economic Organizations (Keidanren) which opposes break-up.

An important element in the government's thinking will be the effect of break-up on the price of NTT shares. The government has been selling off NTT shares to the public and using the proceeds to help pay off the national debt and finance public works construction. But after rising to a high of about ¥3 million (about \$20,000) per share the price has plummeted to ¥1.4 million and the next sale of government-held shares has been postponed.

David Swinbanks

AIDS TREATMENT

'Parallel trick' for ddI

Washington

THE announcement that dideoxyinosin (ddI) will be made available to AIDS patients outside the current clinical trials is a victory for AIDS activists, who have been fighting for early access to drugs working their way through the drug-approval process of the US Federal Drug Administration (FDA). But the victory is tempered by signs that the drug may be more toxic than originally reported.

Results from the recently completed 'Phase 1' safety trials show that ddI, a nucleoside analogue that blocks HIV (human immunodeficiency virus) replication, may be toxic at higher doses, causing nerve damage to the feet and damage to the pancreas. Researchers at the National Institute of Allergy and Infectious Diseases are now ready to begin 'Phase 2' trials, in which the drug's efficacy will be compared to that of AZT, still the only AIDS drug approved for widespread use. Fewer than 30 patients took part in the phase 1 trials, but more than 2,500 will take part in phase 2. At the same time, ddI will be made available to patients who cannot tolerate AZT, or for whom AZT has been ineffective, through a programme administered and funded by Bristol-Myers, the manufacturer of ddI.

With this announcement, the FDA took the first steps towards the much-publicized 'parallel track' mechanism for simultaneous drug testing and distribution. But it remains to be seen whether widespread distribution of ddI will reduce participation in the clinical trials, and there is doubt too over the ethics of approving a drug about which information is still scarce and which may cause serious toxic side-effects.

Christine McGourty

Time to call Japan

Tokyo

A PRICE war may be about to begin in Japan's international telecommunications market, only recently opened up to competition. On 1 October, two new telecommunications companies, International Telecom Japan (ITJ) and International Digital Communications (IDC), began international telephone services with rates 23 to 40 per cent lower than those of the former monopoly Kokusai Denshin Denwa (KDD). But just before the services began, KDD announced new rates, effective from 1 November, that are almost as cheap as those of the new companies. KDD had already reduced its rates considerably in anticipation of competition.

The new companies were at the centre of a trade dispute with the United Kingdom and the United States two years ago, when the Japanese government tried to block participation of Britain's Cable and Wireless and other foreign companies in IDC. One concern of the government has been that establishment of the new companies could result in "excessive competition". Both companies have said they will offer rates considerably cheaper than those of KDD, and a spokesman for IDC says further price reductions are under consideration.

David Swinbanks

PERESTROIKA

Selling ideas at the fringe of science

Irkutsk, Siberia

Perestroika has had at least one disconcerting consequence: the emergence in the Soviet Union of fringe science as a force to reckon with. There is now, for example, a cooperative of scientists based in Leningrad whose income is sufficient to support a staff of 20, mostly on the basis of contracts with industrial enterprises and collective farms.

One of the founders of the cooperative, Anatoly Bondarev, once a field theoretician at Leningrad, was in the Baikal district last week with his Irkutsk-based colleague Eugene Mazhous, urging the value of their techniques in insulating Lake Baikal from pollution. They offer activated water as a way of reducing pollution from industrial enterprises such as oil refineries and extra-sensory cognition as a way of increasing the yield of agricultural crops.

Bondarev says that activated water, made by passing an alternating current through ordinary water, has the property of neutralizing pollutants that may be dissolved in it. As to how activated water differs from ordinary water, he says that its molecular structure retains a pattern of electric dipoles of the kind responsible both for the structure of polywater (a Leningrad invention) and the 'memory of water', coupled with the name of Benveniste.

The agricultural application of extra-sensory phenomena is more shadowy, but the starting point is the argument that a farmer about to sow his seeds for the year ahead will first seek to bring himself and his seeds into harmony with the environment in which the seeds must grow. Extra-sensory processes can enhance the degree of harmony, it appears. Bondarev and Mazhous say that experiments have shown that crop yields can be increased by 30 per cent even when the use of fertilizers and other chemicals is discontinued.

Publication seems to be a sore point. Bondarev says that "before *perestroika*, our Academy of Sciences was very sceptical of our ideas, and prevented their publication". Now, with the opening of the market in ideas, the cooperative, in the process of being separated from a larger organization called ORKON, is free to make what deals it can with industrial and agricultural enterprises. Bondarev says his cooperative has the support of the Irkutsk Executive Committee, an organ of the regional government, and that talks have been opened with the government of Bolivia about the application of the techniques in Latin America.

The cooperative also believes it will be able to exploit cold thermonuclear fusion. Bondarev says he has several ideas as to how cold-fusion processes might function, but that the cooperative will probably not follow the route chosen by Fleischmann and Pons.

John Maddox

PRIZES

Nobel for oncogenes

London

FOR their discovery that the oncogenes of animal tumour viruses are derived from cellular genes (proto-oncogenes), J. Michael Bishop and Harold Varmus of the University of California, San Francisco (UCSF), have won this year's Nobel Prize in Physiology or Medicine.

In the early 1970s, the prevailing 'oncogene hypothesis' of Robert Huebner and George Todaro postulated that the retroviral-like genes carried in the genomes of most mammalian cells were normally silent but, when activated by carcinogens, contributed to the formation of tumours. A minority view, put forward in particular by Steve Martin, Robin Weiss and Peter Fischinger, held that it was normal cell genes that were activated and were also the origin of the oncogenes of retroviruses.

The seminal experiments supporting the minority view were published in 1976 by Bishop and Varmus, with Dominic Stehelin and Peter Vogt (*Nature* 260, 170-173; 1976). In it, they provided evidence that DNA closely related to the *src* oncogene of avian sarcoma viruses is present as part of the full complement of chicken genes, and stated that they were testing whether this DNA represented a gene that was involved in either the normal regulation of cell growth and development or in carcinogen-induced tumour formation.

A huge accumulation of data since 1976 has generalized the initial finding; almost every tumour virus oncogene is derived from a normal cellular gene. More importantly, these proto-oncogenes are active in normal cells and their products are, indeed, involved in cell signalling, growth and differentiation.

The significance of this conclusion in terms of human cancer was uncertain for some years. But since the early 1980s it has become increasingly clear that the mutation of abnormal activation of certain cellular genes, including many of those that crop up in altered form in tumour viruses, contribute to tumour formation. The first evidence that this was so, emerged when a human tumour gene that was able to induce cancer-like properties in cultured cells turned out to be a mutated version of the oncogenes of the Rous sarcoma virus. Robert Weinberg, in whose laboratory that discovery was made, was tipped in the latest issue of *The Scientist* to share the Nobel prize with

Bishop and Varmus, and must, indeed, count himself as unlucky.

Both Bishop and Varmus were born and educated in the United States. Bishop, who is 53, is an MD who took to virology at the National Institutes of Health before moving to UCSF in 1968, where he became professor of microbiology and immunology four years later. He has been director of the George F. Hooper Research Foundation at UCSF since 1981, and continues to pursue research on tumour viruses and proto-oncogenes. Varmus, who will be 50 later this year, has spent the whole of his professional life, sabbaticals apart, at UCSF. Since 1982 he has been professor of biochemistry and biophysics there, and has programmes of research on oncogenes, hepatitis virus and, most recently, human immunodeficiency virus.

Both Harold Varmus and Michael Bishop are known for their scientific



Prizewinners Michael Bishop and Harold Varmus.

writing. The clarity of expression evident in Varmus's papers doubtless owes much to the fact that he graduated in English before turning to medicine and science. Bishop's inimitable style has oncogenes "unleashed" and "incriminated", and not "innocent bystanders or mere pawns in the tumours where they are found". Their lectures in Stockholm in December should make exceptionally good listening.

Peter Newmark

FLOREY INSTITUTE

New director

Sydney

THE new director of the Howard Florey Institute of Experimental Physiology and Medicine in Melbourne is to be John Coghlan, who is at present deputy vice-chancellor for research at the University of Melbourne.

Coghlan has been deputy director of the institute since 1976 and will succeed Derek Denton, who retires on 1 January next year.

Tania Ewing

Next US tokamak in question

Washington

WITH both sides professing to have the best interests of the US nuclear fusion research programme at heart, the fusion physics community and the Office of Energy Research at the Department of Energy (DoE) are displaying a remarkable capacity to derive opposite conclusions from the same set of facts. Physicists want to start work now on the Compact Ignition Tokamak (CIT), a \$700-million machine that, by creating a self-sustaining burn of deuterium-tritium plasma confined in a powerful magnetic field, will give them the experience they need to design an energy-producing fusion reactor. But Robert Hunter, DoE's director of energy research, believes that CIT may well fail in its goal of achieving plasma ignition and wants to put CIT to one side while some outstanding problems in plasma physics are resolved.

Enlivening the debate is an unconcealed feeling in the fusion community that Hunter wants to hold back the decades-old magnetic fusion effort in order to allow research on inertial confinement fusion, in which deuterium-tritium fuel pellets are made to implode by blasting them from all sides with laser beams, to catch up. To make legitimate this subversive plan, the physicists say, he asked DoE's Magnetic Fusion Advisory Committee (MFAC) to assemble an expert review panel to assess the chances that CIT would reach ignition, but then installed as chairman of the panel Dr Kim Molvig, an erstwhile theoretical plasma physicist from the Massachusetts Institute of Technology whom Hunter knew to be a sceptic.

To such charges Hunter responds that physicists are already guilty of overselling the present showpiece machine, Princeton's Tokamak Fusion Test Reactor (TFTR), which after seven years has still fallen short of achieving energy break-even (a transient plasma burn which releases as much energy as was needed to create it). TFTR director Harold Furth says that this was never an official goal, although he admits that many physicists "got enthusiastic" and declared that TFTR would reach break-even. But Hunter says that in a 1981 article discussing the next generation of tokamaks, Furth gives TFTR an "energy multiplicity" of about two — twice break-even, in other words.

It was against this acrimonious background that the investigations and oversight subcommittee of the House of Representatives Committee on Science, Space and Technology last week found itself trying to make sense of plasma confinement and the validity of empirical scaling laws. After three days of hearings, in which the

subcommittee heard from Furth and other fusion laboratory directors, from Molvig and other representatives of MFAC and the academic fusion community, and from Hunter himself, the entire debate on fusion policy seemed to turn on whether a value of 1.45 for a certain plasma confinement parameter was in accord with expectation or an unjustifiable extrapolation.

The physics of hot plasmas entrained in strong magnetic fields is difficult theoretically as well as experimentally. Experimenters rely on an empirical scaling law that estimates the time for which an 'L-mode' (for lowest quality) plasma of given temperature and density can be held in a given magnetic field.

With fine-tuning, machines such as TFTR and the Joint European Torus (JET) routinely attain plasma confinements better than the L-mode by a factor of two or so. In the present design for CIT, there are two construction phases: in phase I, with low field operation, an enhancement factor of 2.1 would be needed to assure plasma ignition; in phase II, a factor of 1.45 would suffice.

So, the majority of physicists at last week's hearing testified, phase I of CIT would be unlikely to achieve ignition, but in phase II a self-sustaining plasma burn would be almost a certainty.

Only at this rather advanced point in the debate does disagreement set in. In the report of MFAC Panel 22, which Molvig chaired, the statement that an enhancement factor of 1.45 is needed if CIT is to reach ignition is followed by a warning: "without a physics basis for the empirical scaling laws . . . there cannot be great confidence at the present time in obtain-

NETWORKS

Exploring connections

Paris

WITH financial help from the European Science Foundation (ESF), a new network has been set up to foster European research in 'neuroimmunomodulation', a young and controversial field whose subject is the interconnections between the immune and central nervous systems. Research is aimed at exploring anecdotal evidence that, for example, depressive patients are more susceptible to physical illness. According to the proposal sent to ESF, the United States is stealing the lead "because of its ability promptly to mobilize human and material resources to explore new and promising areas of research". An ESF grant of FF590,000 (\$91,000) will be used to organize workshops and discussion meetings, once the network is launched on 15 October.

Peter Coles

ing the performance required for ignition". This single sentence, as much as anything else, allowed Hunter to testify that there is "controversy over the scope and design of the fusion programme" and go on to propose that CIT should be delayed while the underlying physics problems were solved.

But according to Fred Ribe, chairman of MFAC itself, the cautionary statement in Panel 22's conclusions is a "molvigism", of which there were at one time many more. Ribe says that Molvig initially wanted to characterize the whole magnetic fusion programme as being "empirically based" but that a lot of "Molvig stuff" was expunged before MFAC passed the Panel 22 report on to Hunter's office. But Ribe felt that some of Molvig's statements, such as the proviso about the likelihood of achieving ignition in CIT, had to be left in, otherwise Molvig would not have accepted the panel's report.

Subcommittee chairman Robert Roe (Democrat, New Jersey) was clearly frustrated by Hunter's assertion that there was controversy over the CIT proposal, when he had previously heard 11 eminent fusion physicists testify in its favour, with only Molvig against an immediate start on the project. And Congressman David Nagle (Democrat, Iowa) established that Hunter's proposal of Molvig as chairman of Panel 22 was unusual: normally, Hunter would ask Ribe to assemble a panel. Ribe told the hearing that he had "acquiesced" in Hunter's suggestion of Molvig, in part because he was pleased by Hunter's personal interest in the fusion programme.

At the end of the three days of the hearing, committee members seemed perplexed that such a sharp disagreement could emerge when everyone, including Molvig and Hunter, agreed that something like CIT was the required next step, and when Hunter reiterated his belief that fusion ignition by the end of the century was the goal DoE should adopt. Perhaps the most clear distinction between the opposing parties was apparent when Roe asked the physicists before him for their assessment of CIT's chances.

With the exception of Molvig, all agreed that ignition was probable, and that in any case not even if the plasma did not ignite, the yield of knowledge would be invaluable. But Hunter's response is that if CIT is being sold to the Congress and to the public as an ignition machine, and fails to ignite, it will join a list of fusion devices which, like TFTR, have not quite lived up to expectations. CIT proponents worry that if the new machine does not move ahead soon, the US fusion programme will fall seriously behind its Japanese and European counterparts; Hunter's fear is that if CIT turns out to be another near-miss, the setback will be worse.

David Lindley

NATURAL HISTORY MUSEUM

A London dinosaur evolves

London

THE Natural History Museum in London hopes to raise £5 million over the next five years for a new series of permanent exhibitions to take it into the next century. The first, on ecology, is scheduled to open at the end of 1990. The money will come from private sponsorship and will be managed by a development trust, launched on 11 October. And the museum is making wider efforts to change its fusty image: an education trip to Disney World in Florida last month is likely to cause a permanent change in the museum's organization and outlook.

Staff returning from the customer care courses at Disney World speak of lessons learned with the kind of conviction normally associated with religious conversion. But many middle-ranking and junior researchers remain unconvinced.

Unlike Disney World, where customer service is based on a clear set of objectives whose success is a matter of corporate pride, the museum, part of the British Civil Service, has had no clear policy on the care of visitors. Some changes have been made — £8.3 million has been invested in new facilities such as a centre for schoolchildren and a spacious cafeteria but the purpose of the Disney trip was to focus vague feelings of a need for change into a plan of attack.

The fact that 17 people went to Florida AUSTRIAN SCIENCE

at public expense, rather than two or three, has come in for criticism, especially from researchers at the museum. Chris Metcalfe, the museum's head of communications, contends that 17 is a small



number, as more than 100 museum staff members are directly involved in visitor care, and the museum's associate director, John Peake argues that £30,000 spent on the trip will be money well spent if staff can then justify the investment needed to improve visitor facilities.

Neil Chalmers' appointment last year as director (see *Nature* 336, 707; 1988) has

accelerated the pace of change at the museum. Several years earlier, the museum had commissioned design consultants Wolff Olins to make suggestions for improvements. Returning this year for more detailed work, the firm found an antiquated institution full of mutual suspicion and a profound lack of communication between staff members.

The museum itself may be the problem. Designed as a Gothic cathedral to knowledge by Alfred Waterhouse and opened to the public on Easter Monday 1881, the museum behind the scenes is a maze of rooms, passages and locked doors that impedes communication and emphasizes divisions. "The whole institution is about not letting anyone know anything about anything", says consultant Wally Olins, a symptom common to other organizations of his experience housed in Waterhouse buildings, which he describes as "murderous... impossible to work in".

Museum staff take the building for granted but recognize that communication problems are serious. Metcalfe, one of Wolff Olins' original design team, joined the museum last November and acknowledges the suspicion fostered by lack of communication as "a key factor to be managed". But efforts to get people involved in the running of the museum are not helped by a workforce that feels its needs have been ignored for long enough.

Research frustrations too may stem from the fact that nobody has thought about the function of a museum with the deliberation that Disney applies to corporate style. Researchers who feel unrecognized and disregarded need, says Olins, to look outwards: instead of seeing themselves as stewards in a "cathedral with 67 million specimens", they should emphasize their role as a unique team with marketable expertise to offer. But many researchers identify with the "cathedral" image and do not see a need to change.

Even small modifications are proving difficult. A new impressionistic logo of a branching tree and the change of name from the "British Museum (Natural History)" to "The Natural History Museum" have excited controversy, largely because of the presumption implied by the definite article. Researchers do not like the change, despite the fact that some overseas researchers still confuse the museum, in South Kensington, with the British Museum in Bloomsbury, another part of central London.

Such resistance hardly augurs well for Olins' grand vision of grouping the many museums, colleges and leisure facilities of South Kensington into "a beautiful, single campus". But despite the many misgivings expressed by researchers, Chalmers still stands by his commitment to research. "Science is enormously important to us: I can't say that strongly enough."

Henry Gee

First five Doppler laboratories launched

Vienna

AUSTRIAN industry took a step towards improving its access to basic research on 28 September when it launched the first five of an estimated 20 'Christian Doppler Laboratories' in fields of potential commercial interest, ranging from biotechnology to expert systems. The project is sponsored by the Austrian nationalized industry Österreichische Industrieholding AG (ÖIAG), subsidiaries of which produce steel, aluminium and chemicals as well as electronic and mechanical components. The rest of the laboratories will be chosen by 1991.

The laboratories, to be located at universities or existing research institutions, are named after the Austrian physicist and mathematician Christian Andreas Doppler (1803–53), eponymous discoverer of the effect by which relative motion between a source of sound waves and a listener alters the perceived pitch of sound. ÖIAG will give each institution up to 3 million Austrian shillings (\$225,000) in one-to-five-year grants, hoping to attract good young researchers to work with the senior scientists around whom each Doppler Laboratory

will be organized.

ÖIAG chairman Hugo Michael Sekyra pointed out at the opening ceremony that the company, which is still recovering from a bankruptcy in 1986, invests only ASch 2,600 million a year in research despite an annual turnover of ASch 150,000 million. Sekyra wants to increase research spending to ASch 4,000 million over the next five years; corporate research planning will be aided by the participation of Doppler laboratory directors.

The new investment is seen as sorely needed in a country that invests only 1.3 per cent of its gross domestic product in research, much less than the 2.5 to 3 per cent investments of other Western nations. The Austrian government now seems destined not to achieve its 1987 promise to increase research investment to 1.5 per cent by 1990.

ÖIAG's determination to raise its standards was illustrated by Sekyra's stated intention to go outside Austria if necessary to set up Doppler laboratories. Going abroad would be unpopular, but ÖIAG might be forced to do so in fields such as materials science. Steven Dickman

New plan for Mono Lake

San Francisco

MONO Lake, in northern California, is a unique ecological site, with hundreds of migratory bird species attracted to the algae and brine shrimp that thrive in its alkaline waters, and a tourist attraction too, drawing hundreds of thousands of visitors every year to see the spectacular limestone tufa towers as well as the wildlife. But since 1941 the level of the lake has fallen 41 feet, as the city of Los Angeles has drawn drinking water from four of the five major feeder streams, and the increasing alkalinity threatens to kill the lake. In an attempt to help resolve one of California's most bitterly fought environmental battles, a bill signed into law late last month offers Los Angeles up to \$60 million if it can find its water elsewhere.

This rich diversity is threatened by the loss of water; the demands of Los Angeles have led to a halving of the lake's volume and a doubling of its salinity. Partial exposure of the alkali-covered lake bed has, according to some, produced a toxic dust laced with arsenic and selenium.

The new legislation will not by itself save the lake, but was endorsed by all parties in the fight and marks a new and potentially pivotal state presence in the decade-long dispute.

Martha Davis, executive director of the Mono Lake Committee, praised the bill for its provision of an incentive for finding alternative sources of water.

Given Los Angeles' current annual water consumption, it has been estimated that the lake's ecosystem will collapse early in the next century. But the city, which receives about 17 per cent of its water from the Mono Lake stream diversions, disputes this and other conclusions.

Several lawsuits surround the Mono Lake controversy, but all have been suspended while the state of California undertakes an exhaustive review, estimated to be finished in late 1993, of the water licences.

The state's offer of \$60 million is designed to coincide with this effort, and is good only if a compromise solution is reached by January 1994. Although Los Angeles supports the legislation, the money is probably a drop in the ocean compared to what is needed. Mitchell Kodama, manager of Mono Basin issues for the Los Angeles Department of Water and Power, guesses that billions of dollars over the next 50 years will be needed to find new water and power supplies, and says that resolving the issue will be impractical without federal assistance, something that Senator Pete Wilson (Republican, California) is expected to propose soon.

Robert Buderl

Industry must help science

London

SOVIET industry must take responsibility for helping to solve scientific and technical problems, Deputy Premier Lev Voronin told the Supreme Soviet at the end of last month. Presenting the draft budget for 1990, he rebuked industrial managers for having failed to respond to recent economic reforms. The restructuring of the Soviet economy calls for enterprises to channel their profits into the development and introduction of new, more productive technologies. But the managers, Voronin said, are actually showing less interest in this field.

The old system of central planning with its rigid targets imposed financial penalties on enterprises which closed down temporarily for retooling, even if the long-term result was greater productivity and higher-quality output. As a result, at the end of the Brezhnev era, there was an ever-growing time gap between technological development and its innovation in industry, sometimes of 10 years or more. Furthermore, the subordination of the major part of the applied research sector to the various industrial ministries meant that much work was duplicated and made interdisciplinary collaboration between institutes under different ministries virtually impossible. A side-effect of the subordination of these 'branch' institutes to their ministries, and hence to a specific industry, was the extremely narrow professional specialization of young scientists.

Mr Mikhail Gorbachev's solution was to advocate the establishment of 'inter-branch scientific and technical complexes (IBSTCs)' to draw together industry and technological research and to break down the bureaucratic barriers to cooperation. Implementing this proposal has proved a slow task.

The Soviet leadership is still firmly committed to these complexes which, Voronin said, will be responsible for a "substantial volume" of the "state order" for research — that is, the priority research areas still controlled by the central planners. In spite of the delegation of much problem-solving research to the decision of industrial managers, the state sector still has a substantial role to play. During the next year, Voronin said, 59 new testing and pilot-production facilities will be commissioned to "consolidate the material base of scientific organizations fulfilling the state programmes".

Not all Soviet scientists, however, share Gorbachev's enthusiasm for the IBSTCs. For many years, Soviet technologists have found themselves on the losing end of a 'two cultures' debate with the 'fundamental' researchers. Subordination to the industrial ministries, the latter said, has made applied research the "handmaiden

of bureaucracy". In a counter-attack just published in *Priroda*, the popular-science monthly of the Soviet Academy of Sciences, Academician Lev Tauson, director of the Vinogradov Geophysical Institute of the Siberian Branch of the Soviet Academy of Sciences, called for the establishment of a new Academy of Technical Sciences, analogous to the existing agricultural and medical academies. Tauson argued that ministries are interested in short-term solutions to specific problems. An independent technology academy, he said, would cost relatively little to establish, because it would take over the existing 'branch' institutes and the applied science institutes of the Academy of Sciences, which would then be left free to pursue fundamental research. Such an academy would be able to see through promising new developments from the initial concept to pilot production. Unfortunately, he admitted, the idea had little chance of success, because the State Committee of Science and Technology still considers that the Soviet Union's industrial problems can best be solved by concentrating financial support on narrowly target-orientated projects and in making 'academic' science ever more utilitarian.

Vera Rich

NEUROSCIENCE

A new line in 'neurocomputers'

London

At a conference in Moscow, experts in non-linear studies decided last month to establish an international centre in Pushchino, Soviet Union, to coordinate work in the development of neurocomputers. The Moscow workshop, *Neurocomputers and Attention*, was organized by the Academy of Sciences of the USSR and the Centre for Nonlinear Studies (CNLS) of the University of Leeds with the aim of working out a coherent strategy for modelling higher brain functions and designing large-scale neurocomputers.

According to Arun Holden, director of the CNLS, participants at the meeting were attempting to bridge the gap between attention modelling and neurobiology. The Soviets introduced the concept of an 'attentional neurocomputer', and although most Western scientists regarded the idea as a good one, they did not think Soviet technology was sophisticated enough to realize it. The planned international centre was decided upon as a way of combining the different Soviet and US approaches, which Holden believes should greatly speed up the development of a neurocomputer.

Ben Webb

Berlin row threatens support

Munich

AN apparently parochial row in the West German Bundestag (parliament) over research support for West Berlin may turn out to have wider implications. In a dispute over the licensing of a nuclear research reactor at the Hahn-Meitner Institute (HMI), the only federally financed Large Research Establishment in West Berlin, the conservative coalition in Bonn may have found an issue that could split the six-month-old Social Democratic (SPD)-Green coalition in West Berlin's governing body, the Senate.

The newly renovated reactor will serve primarily as a neutron source for physicists, chemists and biologists, mostly from outside HMI. Of the total cost of DM 150 million (\$79 million), 90 per cent was paid by the Research Ministry. In a letter sent on 22 September, West Berlin Governing Mayor Walter Momper (SPD) assured Research Minister Heinz Riesenhuber (Christian Democrat, CDU) that the reactor would be licensed by May 1990, assuming that all technical details (such as signing a contract with the United States for radioactive waste disposal) could be cleared up by then. He said he would be

prepared, if necessary, to issue an executive order (*Sofortvollzug*) to eliminate bureaucratic delays.

But Bundestag member Christian Lenzer (CDU) openly doubted Momper's word, saying he expected the licensing procedure to drag on indefinitely, as in the case of the completed but unlicensed fast breeder reactor at Kalkar. The Bundestag science and technology committee then threatened to cut off the financing of new projects in West Berlin unless the reactor licensing moved ahead. The committee's conservative majority demanded a "clear, reliable course" from the West Berlin government.

Although its rhetoric is critical of science and technology — for example, calling for more studies of the impact of technology on society — the SPD-Green government has not yet taken any strong positions against science. In closing the academy, Momper said the money would be better invested in improving conditions at overcrowded universities. But, with elections a year away, the SPD and the Greens have sent out conflicting messages about issues such as nuclear power.

Social Democrats in the Bundestag

attacked the committee's threat in a tumultuous plenary debate. Edelgard Bulmahn (SPD) charged the conservative parties with "unprecedented interference" in the internal affairs of the *Land* (state) of West Berlin tantamount to "blackmail", which could have "lasting consequences." And the issue could even break up the SPD-Green coalition.

If Momper makes good his promise, it will put Environment Senator Michaela Schreyer of the *Alternative Liste* (AL), the local branch of the Greens, in the unaccustomed and difficult position of having to sign a reactor licence. The distinction between research and power reactors is not seen as relevant by the many AL members who oppose the reactor.

Although HMI welcomed the pressure from Bonn, saying that it was "very useful" that Momper had now set a deadline for licensing, the heavy-handed approach of the conservative coalition in the Bundestag offended West Berlin industry, traditionally supporters of conservative causes. The Chamber of Commerce and Industry (IHK in German) warned that the committee's threat may drive new investment away from the city.

West Berlin is dependent on the Bonn government for most of its research support, as it is, in fact, for 52 per cent of its overall budget. It receives 6 per cent of all federal research funds, although it has only 3.1 per cent of the total population of West Germany. Research, basic and applied, is an economic lifeline for the city.

Steven Dickman

TECHNOLOGY TRANSFER

Adviser learns Japanese

Washington

THE new director of the US Office of Science and Technology Policy (OSTP), Allan Bromley, admitted last week that the system for transferring technology from federal laboratories to industry was beset by "byzantine complexities" and promised that improving it would be high on his agenda for the 1990s. His plans include setting up a new industrial technology section within the OSTP with a powerful presidentially appointed director, and he suggested that both government and industry had a lot to learn from the Japanese.

Under a grilling by members of the House of Representatives subcommittee on regulation, business opportunities and energy, Bromley said he was "committed to emphasizing the 'T' in OSTP" much more than his predecessors, and suggested that the United States adopt some features of the Japanese system, the success of which he attributed to "putting basic researchers, development engineers, manufacturing engineers and materials experts in groups, stirring briskly, and then assuring them of stable support in their pursuit of broadly specified goals".

The features that make the Japanese system work, he said, are: the targeting of key technologies for special attention; a long-term commitment of money and

people "even if results are slow in coming or don't come at all"; cooperation between industry, government and universities; collaboration between basic researchers, development engineers and experts in manufacturing and materials; and the training of a large cadre of highly-specialized "supertechnicians".

But in the United States, Bromley said, individual companies waste expertise, time and money reinventing basic technologies, and the division between research and manufacturing means that US-designed products may be intrinsically difficult to make, and thus expensive. And the lack of "supertechnicians" means US companies must often go to Japan for samples of materials invented and developed in the United States. Bromley stressed, however, that he did not advocate "slavishly copying" the Japanese model, but rather selecting parts of it.

The consequences of the poor US system were laid out in a report by the subcommittee staff, which calculated that the return to the government in 1988 from licensing of federal inventions and discoveries amounted to only 0.00005 per cent of the research investment. It also complained that federal laboratories are ignoring new technology transfer laws and called for a top-level administration executive to take responsibility for monitoring

their technology transfer efforts.

The report was endorsed by Robert Brumley, former general counsel for the Commerce Department, who said that "the problem with the federal technology transfer program is that no-one understands it".

While working at the Commerce Department on technology transfer related to the space station, Brumley attempted to invoke the Federal Technology Transfer Act of 1986 to force the government to increase its technology transfer efforts and found that "not one member participating either from the National Aeronautics and Space Administration, the Department of Defense, the State Department, the Office of Management and Budget or the White House" was aware of the provisions of the act.

James Ling, a science adviser to former president Ronald Reagan, blamed federal officials for taking the attitude that "every company is trying to 'rip off' the government". Unless the situation improves, he proposes taking radical steps: "If we can't get more out of these activities we should scale them back or even shut them down", because they are tying up resources that could be better used in the private sector.

Christine McGourty

Problems at Max Planck

SIR—I would like to respond to your article on the structural problems of the Max Planck Society (MPS) (*Nature* **340**; 335; 1989).

The MPS does not consist only of directors and slaves; there is also a sizeable number of middle-rank employees (equivalent to tenured associate professors in US universities) whose scientific reputation is independent of the director. They run their own groups and programmes, with much of their support coming from outside grants. Many of them have come back to West Germany from good overseas posts, hoping to use their positions either to move up within the MPS or to obtain a university professorship. To the frustration of many of them, (1) the low level of institutional recognition given to them makes a career within MPS highly unlikely, and (2) the congestion at the top of West German universities, both in terms of available professorships and resources, seriously limits their flexibility. At present, these group leaders face the same dark future as other employees left behind when a director retires.

Many of the current problems of the MPS could be solved if the society would develop an attractive career system for younger scientists similar to that of tenure-track positions in US universities. The independence of scientists should parallel their ability to carry out independent research, their motivation should be fostered by a reasonable outlook and institutional recognition, and the MPS should develop a system of internal competition for resources. A fresh approach is necessary in which the present privileges and endowments of MPS directors are more reasonably divided between all the members of the MPS community. The hope is with the new generation of directors but a chorus of voices is required in order to be heard.

ALFRED MAELICKE

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SIR—The article on the Max-Planck Society (MPS) highlights one of the structural problems this society has. In addition, there is at least one other problem. The MPS has chosen to employ particularly able scientists on tenured contracts equivalent to those of middle-rank professor at the universities. Their number is about 200 and thus similar to that of the directors. Many of them function as group leaders on projects that are independent although often related to that of the director, and always dependent on him for resources. This group of scientists contribute greatly to the reputation of the MPS. Yet, as the

MPS has failed to make provision for such groups as independent units, the society sees their existence as a private arrangement with the director, with the consequence that resources will be cut off when the director retires. These colleagues have been chosen on their scientific merit and in many cases are expected to run a group, so it is a slap in the face that their scientific career in the MPS should essentially be terminated with that of the director. The MPS recognizes as units only departments with directors as heads. Rare exceptions are the groups for junior scientists established on a five-year basis. Although there might have been a justification for this hierarchical structure in earlier days, it is inadequate for a research organization nowadays. It should be adjusted to present-day requirements by giving autonomy to the *de facto* existing mid-level positions. The MPS would benefit from such a change by a decrease in size of departments and the creation of a livelier, more stimulating atmosphere in the institutes.

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Reason to write

SIR—As a scientist and a member of the human rights group Amnesty International, I would like to bring to the attention of the scientific community one of the many human stories that underlie the brutal repression in China in the past several months. *Nature* has reported on the events in China, and on some of the arrests that have occurred in the Chinese scientific community (for example **340**, 174; 1989). Among those arrested in the crackdown was Yang Wei.

Yang Wei was one of the many Chinese students to go abroad in the past ten years, spending several years as a molecular biology student at the University of Arizona. Many of us have had the privilege of meeting such students. As they learned about science, we watched as they adapted to the West and learned about the freedoms we enjoy.

Yang Wei was unfortunate enough to have been at home for a visit during the earlier round of student demonstrations, and he was among those arrested in January 1987 and imprisoned. During his imprisonment he was adopted as a prisoner of conscience by Amnesty International. His two-year prison term ended in January this year, and he was awaiting permission to return to his studies in the United States, and to rejoin his wife, also a student here. These plans came to nothing

on 19 July, when he was arrested again — allegedly for involvement in the recent round of demonstrations.

Any of the thousands of Chinese students abroad could be Yang Wei, imprisoned for speaking out, something we had taught them to do, both directly and by example. Seeking out the truth and speaking it underlie the ideal of scientific enterprise. We cannot abandon our colleagues in China who are now being punished for speaking the truth. One way to remember them is to write to Prime Minister Li Peng (Guowuyan, Beijingshi/People's Republic of China) on behalf of Yang Wei, expressing concern at the reports of arbitrary arrests such as his. We must remind the Chinese leaders that we will not forget the events of June or their aftermath.

MARK PEIFER

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Pitt not Penn

SIR—A leading article on "Conflicts of interest" (*Nature* **340**, 664; 1989) commented on "the dispute that has now come to light at the University of Pennsylvania". However, it is stated on page 668 that the dispute on conflicts of interest is at the University of Pittsburgh. Please note that the University of Pennsylvania, named for William Penn, is the one founded by Benjamin Franklin in 1740 and is beginning its 250th anniversary celebrations. The University of Pittsburgh, which has the dispute, was named for William Pitt the Elder, and was chartered in 1787. Although it is a university in Pennsylvania, it is not the University of Pennsylvania and is more than 300 miles away from here.

ROBERT E. DAVIES

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Women in physics

SIR—Only 130 out of 1,302 doctorates granted in physics in the United States in 1988 went to women (*Nature* **340**, 417; 1989).

In the same issue, you wrote "By now, of course, every schoolboy knows that the quintessential point in space-time is the occasion of the Big Bang . . ." (*Nature* **340**, 425; 1989).

Thank you for pointing out that the underrepresentation of females in physics begins at an early age.

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Baikal centre takes step forward

The project to found an international research centre on Lake Baikal took another modest step forward last week, but there remains a great deal to be done.

Listvianka (on Lake Baikal)

How do you set about founding an international research centre the like of which has not been seen before? That is the question with which a group of Soviet and overseas limnologists wrestled last week. They got as far as signing a declaration of intent, but the hard work still lies ahead.

The project to found an international research centre on the shores of Lake Baikal is nevertheless breathtaking in its audacity. The idea is to create within the previously closed Soviet Union a centre in whose scientific management foreign members will have the decisive voice. Most probably, the director will also be from overseas, although there will be a Soviet executive director who will function as a legally responsible accounting officer.

The prime mover is Dr M. A. Grachev, a molecular biologist who is now director of the Limnological Institute of Irkutsk, the nearest city, but he has the solid backing of Academician Valentin A. Keptug, president of the semi-autonomous Siberian branch of the Soviet Academy of Sciences. The Siberian branch has offered a down payment of 5 million roubles and at least matching funds thereafter.

Lake Baikal's claims on public attention are well-known (see, for example, *Nature* **329**, 802; 1987 and **337**, 111; 1989). It holds perhaps a fifth of the total fresh water on the surface of the Earth, and appears to be in its pristine state, a couple of paper-pulp plants on the southern shore notwithstanding. Unlike most other deep lakes, Baikal retains oxygen even at its greatest depth of 1,700 metres. There is a staggering array of endemic flora and fauna, including a population of 20,000 seals adapted not merely to fresh water but to life on the surface of the couple of metres of ice covering the lake in winter. Yet the origins of those forms, as of the lake itself, remain mostly a puzzle.

Last week's proceedings fell into two parts. During waking hours, there was a symposium planned as a survey of the opportunities Lake Baikal offers, happily coinciding with the centenary of the birth of G. Yu. Vereshchagin, one of the pioneers of research at Lake Baikal after whom the largest of the lake's seven research vessels is named.

Evenings were given over to politicking, mostly the discussion of a draft charter for the centre and the draft of a more detailed

agreement that will determine how the centre operates. There was the usual flurry of late-night and inexpert typing. The general opinion seems to be that it is too soon to draft a final constitution for the centre. There is some hope that that may be possible by next May (when the ice will have gone), but that may be frustrated.

But there is a sense in which the centre is already a going concern. Almost a score of joint projects have been mounted in the past two years under the aegis of the Limnological Institute. The usual pattern is that there will be an agreement to carry out some investigation between an overseas group and one recruited in the Soviet Union, not necessarily from Irkutsk. The benefits for overseas groups are considerable — the Soviet side provides free travel from Moscow and free accommodation, while the Limnological Institute puts its seven research vessels at the disposal of the international groups.

So why not let these arrangements multiply naturally? That may be what will happen. But the Soviet side would like to see the programme grow faster if that can be done respectably. Despite a century's desultory research, much of it published at huge length in Russian-language monographs, too little is known about the present state of Lake Baikal. There is even room for argument, for example, about the water-balance of the lake, with thousands of inlets but only one outlet, the Angara; some believe that a credible water balance requires subterranean input.

The origin of Lake Baikal, more than 700 km long, is similarly in dispute. Although it closely resembles the great Rift Valley lakes of East Africa, there are only the sketchiest signs that it is part of a larger system of faulting and subsidence. But mounting a deep seismic survey of the whole Baikal region will be a formidable undertaking, requiring years of effort.

Meanwhile, there are more practical difficulties to contend with. The Limnological Institute, the centre-in-waiting, is more than 70 km away. There is an urgent need for workshop and laboratory space near the lake itself, together with dormitories in which researchers can sleep. So the Siberian branch hopes that it can persuade its potential research partners to contribute towards the cost of these facilities, perhaps between \$15 million and \$20 million. One scheme floated last week is that there should be three classes of

members, with so-called founder members defined as those able to contribute \$500,000 of capital at the outset being alone entitled to sit on the board of management of the centre. One unique condition attached to these contributions is that, if the centre should cease to operate, the funds would be returned.

The formula does not appeal to everybody. One difficulty is that university research groups could not contribute funds on such a scale out of their initial contributions from their governments or government grant-making agencies, which would be unwilling to delegate the prestige of making such a large and conspicuous grant to a mere grant-applicant. And that would lead to an international treaty between governments rather than to a research-led organization of the kind intended. Koptag says that "we have some experience of this kind of organization", but it may be a more serious handicap that the Soviet Union does not appreciate that the apparently uniformly well-equipped research enterprise in the West rests on tooth-and-nail competition for funds.

There are other problems, talked about less openly last week. One danger is that people may be tempted to repeat on Lake Baikal studies already carried out elsewhere, without much thought of what they mean. Grachev, for one, would prefer that the final constitution of the international centre were delayed than that it should spawn a flood of second-rate science.

So there seems enough genuine enthusiasm to ensure that something will have happened by next May. The letter of intent commits its signatories only to do what they can to realize that goal. The signatories include representatives of several US research groups, two delegates from China (who came by train), and people from Canada, Sweden and West Germany. The Royal Society (of London), which has a committee working on the project, was formally represented by a delegation, but the smaller Belgian delegation signed an interim agreement with the Siberian branch for a comparative study of Baikal and the East African rift lakes, chiefly Tanganyika and Malawi, on the understanding that it will be subsumed in the agreement to found a centre when, formally, there is one. It will be a shame if this enthusiasm comes to nothing.

John Maddox

Immunotherapy by peptides?

Charles A. Janeway

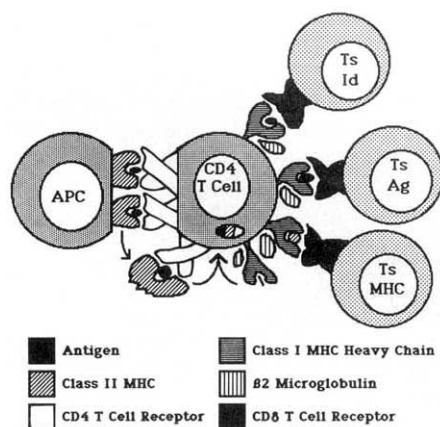
AUTOIMMUNE diseases occur when T lymphocytes become persistently activated by self antigens. Like all antigens recognized by T cells, these consist of a peptide fragment, which is held in the cleft of a major histocompatibility complex (MHC) molecule (class I MHC in the case of CD8 T cells and class II in the case of CD4 cells). Thus, one approach to therapy of autoimmune disease is to provide a substitute peptide that can remove the stimulus that is turning on the T-cell response. An alternative approach is suggested by recent studies showing homogeneity in the receptors of the T cells generating several autoimmune diseases. Peptides based on the sequence of the receptor on such T cells might immunize an animal against autoimmunity, as shown in the report of Vandenbark *et al.* on page 541 of this issue¹. These contrasting approaches to peptide immunotherapy raise several interesting questions about the autoimmune process and its specific inhibition.

Currently, one of the best characterized autoimmune diseases is experimental allergic encephalomyelitis (EAE), induced in mice or rats by injection of myelin basic protein (MBP) in strong adjuvants. In H-2^m mice, the disease is mediated by CD4 T cells recognizing the acetylated amino-terminal nonapeptide (Ac1-9) of MBP bound to the class II I-A^m molecule; in rats, a different peptide of MBP is recognized. T cells that can experimentally transfer EAE from one animal to another have been shown in both mice and rats to have receptors whose variable domains are encoded by a very limited, and highly homologous, set of V-region gene segments. With the detailed characterization of both the autoantigenic ligand and the T-cell receptor that recognizes that ligand in EAE, it has been possible to design rational peptide-based immunotherapies. Initial results, although promising, raise more questions than answers.

Two groups have used synthetic peptides to explore the recognition of Ac1-9 by disease-inducing or encephalitogenic cloned T-cell lines^{2,3}. Both find that peptides modified at position 4 can bind class II MHC molecules much more strongly than the native peptide, potently stimulating encephalitogenic clones but, surprisingly, not inducing disease *in vivo*. Indeed, co-immunization with the native and amino-acid-4 modified peptides prevents the normal induction of EAE. The same is true of peptides modified at both amino acids 3 and 4, which bind class II MHC molecules strongly, but no longer interact with the T-cell receptor on encephalitogenic clones.

Whereas the rationale underlying this

approach is to inhibit binding of auto-antigenic peptides to class II MHC molecules, there is presently no evidence that this is the mechanism by which the EAE is prevented. It is also possible that the peptides are pre-empting the immune response by generating suppressor T cells or CD4 T cells of a distinct and non-pathogenic functional type, particularly in the case of the position-4 substitutions that retain antigenicity but lose the ability to induce disease. The class of CD4 T cells



An antigen-presenting cell (APC) stimulates a CD4 T cell, whose T-cell receptor binds the peptide—class II MHC complex, somehow detaches it from the APC and internalizes it via endosomes, where the class II MHC molecule and the T-cell receptor are degraded, and the peptide is released. After binding to class I MHC molecules, which are believed to recycle through endosomes in T cells, the peptide is in turn presented to CD8 suppressor T cells specific for peptides derived from the T-cell receptor (Ts Id), the MHC molecule (Ts MHC) or the antigen itself (Ts Ag). The CD8 T cells serve to regulate the activity of the CD4 T cell. Immunization with T-cell receptor peptide would stimulate the Ts Id in this scheme.

activated in several *in vivo* immune responses has been shown to have a strong influence on biological consequences^{4,5}.

This form of peptide immunotherapy is, at least in concept, essentially passive. Because the exogenous peptide must compete with endogenous peptides for the MHC peptide-binding site, it must be continuously supplied to avoid induction of the autoimmune response.

An alternative approach is deliberately to vaccinate against the autoreactive T cell. This approach was pioneered by Irun Cohen and co-workers at the Weizmann Institute, who in 1981 showed that rats given attenuated encephalitogenic cloned T cells become immunized against EAE⁶. They postulated that the target for this vaccination was the T-cell receptor. The recent demonstration in both rats and mice that encephalitogenic cloned T-cell

lines have very restricted receptor variability, and the recent successful immunotherapy of EAE with monoclonal antibodies to shared T-cell receptor epitopes, are consistent with this view⁷. Following this lead, Vandenbark *et al.*¹ and Howell, Brostoff and colleagues⁸ have now successfully prevented EAE in rats by immunizing them with synthetic T-cell receptor peptides emulsified in adjuvant. Interestingly, while Vandenbark *et al.* use a peptide from the CDR2 region of the receptor β -chain, Howell *et al.* use a peptide from the VDJ β region. Vandenbark *et al.* have gone on to show that CD8 T cells that respond to the receptor peptide presented by class I MHC molecules can transfer protection against EAE. These studies show quite conclusively that suppressive T cells can turn off the autoimmune response that causes EAE.

This active approach of turning off EAE by T-cell-receptor peptide immunization — like the passive approach that uses blocking peptides to prevent the autoimmune response from being turned on — raises numerous questions. The first of these is whether the active approach will be feasible in other autoimmune diseases. To be successful, the T cells causing the disease must be very homogeneous. There is evidence that this condition is met in experimental autoimmune diabetes⁹ as well as in human rheumatoid arthritis¹⁰ and multiple sclerosis¹¹, for which EAE is considered a model, although in no case is this settled. In addition, the precise structure of the T-cell receptor must be known in each disease. If these obstacles can be overcome, this form of active immunotherapy would be highly attractive, as it requires only a vaccinating dose of peptide and not continuous treatment, and it offers the possibility of specific dominant suppression of a continuing immune response, which is always a difficult goal to achieve.

Perhaps the most interesting question raised by the present experiments is the mechanism by which the suppressive T cells recognize their specific target. Most peptide immunogens stimulate the CD4 T cells that recognize antigen presented by class II MHC molecules, but Vandenbark *et al.* have stimulated CD8 T cells that are class I MHC restricted. The target cell is presumably recognized with the same specificity. While it has been shown — most dramatically by Townsend *et al.* in a recent article in *Nature*¹² — that exogenous peptides can bind effectively to class I MHC molecules, the stimulation of CD8 T cells by extrinsic peptide immunization has been a rarity. This suggests that novel mechanisms may be operating in the Vandenbark *et al.* system.

T-cell-receptor peptides could arise by intracellular degradation and associate with class I MHC molecules. A more novel and interesting explanation for

these results is that T cells, unlike conventional antigen-presenting cells, internalize class I MHC molecules via the endocytic pathway¹³, where they can bind peptides generated in endosomes and then present them at the T-cell surface, as shown in the figure. Activated T cells could present peptides derived from three structures: the antigenic peptide itself, the class II MHC molecule, or the T-cell receptor. As also indicated in the figure, activated T cells can stably and specifically bind MHC molecules derived from the antigen-presenting cell¹⁴.

This postulated mechanism has several features of interest. First, the target of suppression would only appear on activated T cells undergoing a specific immune response, the only legitimate targets of suppression. Second, this mechanism obeys the known rules of T-cell recognition of antigen, although it postulates a behaviour of class I MHC molecules on T cells that is not yet proved. Third, this mechanism of suppression could be invoked to explain reported examples of suppression that are specific for antigen, MHC or idio type. Fourth, it suggests a novel explanation for the enigma of the I-J determinant that is so central in studies of suppressor T-cell function¹⁵. If I-J consists of a peptide derived from a class II MHC molecule bound to a non-polymorphic class I MHC molecule similar to TL or Hmt¹⁶, then the specificity of the determinant would be controlled by class II MHC even though the macromolecular component is not a class II MHC product. An antibody to a peptide-MHC complex has been defined¹⁷, suggesting that this model is possible. Furthermore, the recent structural analysis of I-J by Tada and colleagues is consistent with I-J being a peptide complexed to a class I MHC product¹⁸. Finally, one may ask whether the paradoxical behaviour of the Ac1-9 peptide analogues could be explained by a similar mechanism. Are the peptides presented by class I MHC to activate suppressor cells that then inhibit the

activity of encephalitogenic clones?

The crucial issues for the future are clear. First, can either type of peptide immunotherapy inhibit disease? Second, is there a homogeneity of T-cell receptors in man? Third, will such therapies inhibit essential beneficial immune responses? Fourth, will the MHC genotype of the individual affect the peptide that must be used? These questions must be answered before we can look forward to peptide

immunotherapy or immune prophylaxis of autoimmune disease. Will the physicians treating autoimmunity in the future echo Shaw's caricature of Sir Almroth Wright, murmuring "stimulate the suppressors" as they administer their vaccines? Only time and a great deal of work will tell. □

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OROGENY

Crustal evolution in the Andes

Marjorie Wilson

SINCE the advent of plate tectonics it has generally been accepted that active continental margins (sites where an oceanic plate is subducted beneath a continent) represent important loci for the growth of new continental crust. For net growth to occur, new magmatic material, derived by partial melting of the Earth's upper mantle, must be added to existing crust in the form of surface volcanics, intra-crustal intrusions or underplated basic material. J.F. Miller and N.B.W. Harris now present in *Geology* (17, 615-617; 1989) an interesting synthesis of neodymium isotope data for igneous rocks generated during 300 million years of subduction-related magmatism in the Central Andes in an attempt to distinguish periods of crustal growth from those of crustal reworking. Their approach relies on the assumption that, under certain favourable circumstances, the Nd isotope composition of an igneous rock at the time of its formation (expressed as an initial ratio of ¹⁴³Nd/¹⁴⁴Nd or ϵ_{Nd} value) can fingerprint the source characteristics of the parent magma. Although the approach is neither new nor innovative, the authors provide data fundamental to our understanding of both the rates and mechanisms of crustal growth throughout geological time.

It is now generally accepted that the

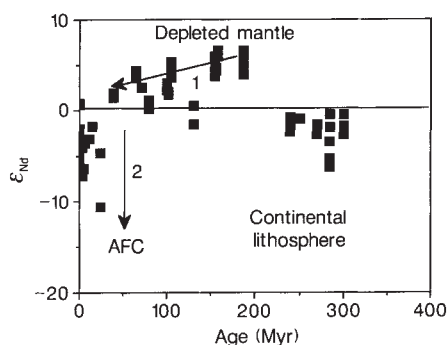
main source of basaltic magma in all subduction-related tectonic settings (both island arcs and active continental margins) is the wedge of depleted asthenospheric upper mantle overlying the slab of subducted oceanic lithosphere. Once generated, these primary magmas undergo variable and complex processes of fractionation and contamination during their passage through the lithosphere (crust plus mantle) en route to the surface. Over the past 300 million years (Myr), the depleted mantle reservoir has been characterized by a rather restricted range of ϵ_{Nd} values from about +7 to +10 (see graph). In contrast continental crustal rocks have highly variable values (mostly negative, by as much as -30) that become progressively more negative as the age of the crust increases. If these two reservoirs of Nd were the only ones involved in the magma generation process then the interpretation of the isotope characteristics of these central Andean rocks, in terms of distinguishing periods of crustal growth (by addition of mantle-derived magmas) from those of crustal reworking (by partial melting of pre-existing crust), would be comparatively straightforward.

Unfortunately in the active-continental-margin tectonic setting, potential interactions between the primary magmas

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REASONS

Mt Huayna Potosi, Cordillera Real of the central Andes, Bolivia: complex evolution at work.

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Neodymium isotope variations (ϵ_{Nd}) of central Andean igneous rocks as a function of age. Miller and Harris attempt, in their new study, to relate the values of ϵ_{Nd} , which compares the initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratio of a sample with that of a chondritic reservoir (CHUR), to changes in the sources of Andean rocks and so to trace the Andean evolution. (Redrawn from *Geology* **17**, 615–617; 1989).

and enriched subcontinental lithospheric mantle components (which can have ϵ_{Nd} values overlapping those of crustal rocks) further complicate the issue. This had led to a degree of controversy between the various groups currently studying Andean magmatism, as exemplified in recent articles by G. Rogers and C.J. Hawkesworth (*Earth planet. Sci. Lett.* **91**, 271–285; 1989) and W. Hildreth and S. Moorbath (*Contr. Miner. Petrol.* **98**, 455–459; 1988).

Palaeozoic granitic intrusions (more than 240 Myr old) from the central Andes are characterized by negative ϵ_{Nd} values (see graph) which Miller and Harris interpret in terms of a crustal reworking event with little net crustal growth. In contrast, at the onset of the main mountain-building phase (orogeny) of the Andes (less than 200 Myr ago), ϵ_{Nd} values for a wide compositional spectrum of volcanic and plutonic rocks become strongly positive (+4 to +7) suggesting an increasing asthenospheric component to the magmatism, consistent with crustal growth. However, from 180 to 40 Myr ago, ϵ_{Nd} values decline steadily (trend 1 in the graph) followed by a much more rapid decrease in the past 30 Myr (trend 2), which may suggest increasing degrees of crustal reworking.

Rogers and Hawkesworth, however, prefer a different interpretation for trend 1, involving the progressive mobilization of late Proterozoic mantle lithosphere beneath the arc as magmatism migrated eastwards across northern Chile with time. If correct, this would imply that all samples with ϵ_{Nd} values from +6 to 0 could represent new magmatic inputs to the crust. Miller and Harris concur with Rogers and Hawkesworth that trend 2 is most easily explained in terms of high-level assimilation and fractional crystallization (AFC) processes, postdating a period of rapid thickening of the crust to 70–80 km caused by crustal shortening. These involve the assimilation of crustal rocks (of contrasting isotopic composition)

by the rising magmas, which then cool, crystallize and differentiate.

Hildreth and Moorbath present extremely convincing arguments that ϵ_{Nd} variations, as large as those observed in trend 1, along a segment of the Quaternary (post 1.6 Myr) volcanic front in the southern Andes (33–37° S), can be explained by a model of progressive crustal contamination related to increasing crustal thickness (and possibly crustal age) from south to north. The contamination is envisaged to occur in MASH (melting–assimilation–storage–homogenization) zones at the base of crust. In such zones mantle-derived basaltic magmas mix with acidic partial melts of lower crustal rocks, generating a distinctive base-level isotope signature at each volcano. High-level AFC processes may then further modify the isotopic composition of the ascending magmas to lower ϵ_{Nd} values. It seems reasonable that a similar model could equally be applicable to the central Andes in which the decreasing base-level ϵ_{Nd} values along trend 1 reflect increasing crustal contamination in MASH zones, as a consequence

of progressive crustal thickening over the past 200 Myr. However, Nd isotope data alone clearly cannot preclude the involvement of enriched lithospheric mantle.

In general, although their article is thought provoking, I feel that Miller and Harris tend to oversimplify the problem of identifying new mantle-derived additions to the continental crust. One important point that seems to have been overlooked is that the strong evidence for an increased crustal Nd component in the younger magmas in no way indicates a declining magmatic input to the system. The crust still grows by the addition of basic magma to lower-crustal MASH zones but this growth is not necessarily reflected in the Nd isotope composition of the erupted magmas. Clearly much more work needs to be done to characterize the geochemical and isotopic variations of Andean magmatism in space and time before we can realistically hope to model crustal growth processes. □

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ANTIBODY TECHNOLOGY

Will dAbs challenge mAbs?

Penelope Austin

HAVING been unable to profit from the advance that made monoclonal antibody (mAb) production from hybridomas possible¹, the UK Medical Research Council (MRC) has made sure of claiming its rights to a discovery that may make hybridoma technology less important than before. On page 544 of this issue², Ward *et al.*, from the same Laboratory of Molecular Biology whence hybridoma technology emerged, describe how bacteria may be employed to produce antigen-specific reagents. The new approach depends in part on the ability to clone a diverse repertoire of rearranged immunoglobulin heavy chain variable (VH) genes, and express them efficiently in bacteria^{2,3}. But its success is largely due to the surprising discovery that many VH domains bind antigen with good affinity in the absence of their partner light chain (VL) domains.

Ward *et al.* stumbled upon this unexpected phenomenon while investigating the antigen-binding interactions of the anti-lysozyme antibody D1.3. The crystal structure of D1.3 shows that both heavy and light chains make significant contacts with lysozyme⁴. Although there is some asymmetry, the light chain contributes over 40 per cent of the antigen-binding surface, and few would have predicted that it is not required. In fact, isolated D1.3 VH domains prepared from bacteria bind lysozyme with an affinity only tenfold

lower than that of the parent antibody, indicating that the net contribution made by the κ -chain chain to the energetics of binding is small.

With polymerase chain reaction (PCR) primers designed to amplify and clone rearranged VH genes, and an expression vector that directs the secretion of isolated VH domains in *Escherichia coli* already in hand⁵, Ward *et al.* were well placed to explore the possibility that D1.3 was not unique in this respect. After demonstrating their ability to use the primers to clone an extensive repertoire of VH genes from the spleen genomic DNA of immunized mice, they made and screened two libraries, one from a mouse immunized with lysozyme, the second from a mouse immunized with keyhole limpet haemocyanin (KLH). VH domains with antigen-binding activity were not hard to find (21 lysozyme-binding and 2 KLH-binding supernatants out of about 2,000 for the lysozyme library; 14 KLH-binding and 2 lysozyme-binding supernatants out of about 2,000 for the KLH library), and for the two lysozyme-specific clones analysed in more detail, the affinity of binding was similar (in the 20 nM range) to that seen for D1.3.

As well as presenting evidence that antigen-specific VH domains are common, these experiments lay the foundation of a powerful new technology. Antigen-binding activity can be detected within as little as 3

days of harvesting the spleen of an immunized animal, and the need for tissue culture is avoided. In principle, the VH domains could be used as building blocks and further selection — for higher-affinity antigen binding — could be imposed after mutation or co-expression with light chain domains.

With the patent application filed away, the MRC is discussing potential applications with a number of companies. For some purposes, such as the rapid clearance of toxins from body tissues, dAbs may have special advantages. For projects, such as the reconstruction of high-affinity antibodies for use in human therapy or the design of catalytic antibodies, dAbs should prove invaluable as building blocks. Whether dAbs will compete with mAbs for more routine procedures, such as the affinity and purification of proteins, will depend on cost. In theory dAbs could be produced more cheaply, but problems of 'stickiness' would need to be engineered out, and production levels raised.

The interest in commercial development shown by the MRC seems a far cry

from the days when the National Research Development Council, the only channel through which MRC scientists could then exploit their inventions, wrote of Köhler and Milstein's original monoclonal antibody paper, "It is certainly difficult for us to identify any immediate practical application which could be pursued as a commercial venture". The new advance may even provide an opportunity to gain some ground lost. Greg Winter, senior author of Ward *et al.*, is enthusiastic about the new research and patent opportunities. Ironically, he also sees dAbs as potential "patent busters", able to undermine many of the patented applications of monoclonal antibodies now in force. □

Penelope Austin is an Assistant Editor of Nature.

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CELL BIOLOGY

Firm base for basal body DNA

Jeremy S. Hyams

BASAL bodies and their *alter ego* the centriole are amongst the most enigmatic cell organelles¹. They seed the growth of flagella and cilia, they have a complex pin-wheel structure (see figure), they can undergo Jeckyll and Hyde-like inter-conversion at specific points in the cell cycle and, most mysteriously, are able to duplicate themselves in a process that is tightly coupled to, but independent from, the mitotic cycle². The last property has prompted considerable speculation about the existence of basal body/centriole DNA, but a paper from David Luck's group in the latest issue of *Cell*³ leaves no doubt as to its reality.

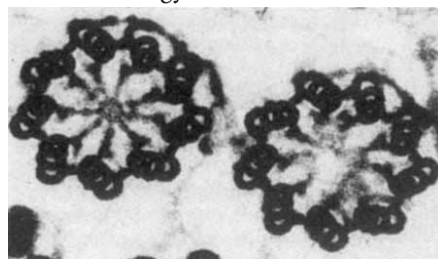
Hall *et al.*³ have studied the biflagellate, unicellular green alga *Chlamydomonas reinhardtii*, a popular model for studies of eukaryotic flagellar function because of the extensive collection of mutants affecting the structure of the axoneme — the flagellar core, which is constructed around nine pairs of doublet microtubules surrounding a pair of single microtubules (the 9+2 axoneme)^{4,5}. Although a few of these mutants are sterile (the flagella of *Chlamydomonas* are also used in sexual reproduction), the remainder show classical 2:2 mendelian segregation in a genetic cross and map to one of the 18 nuclear linkage groups in this organism (*Chlamydomonas* also has a well characterized chloroplast genome, mutations in which show non-mendelian segregation).

One class of flagellar mutants, how-

ever, violates this basic rule. Called *uni*⁶, these strains fail to develop one of the two flagella, with the result that they swim around in circles like a sculler who has lost an oar. The *uni* mutations segregate 2:2 in a cross, but are unlinked to any known chromosomal marker. They do, however, show linkage to a number of other mutants having altered flagellar development, possibly as the result of a defect in some aspect of basal-body function. Analysis of this *uni* linkage group (ULG; also referred to as linkage group XIX⁷) has shown it to be genetically circular⁸.

It has long seemed likely that *Chlamydomonas* ULG would be the best for laying to rest some of the age old arguments regarding the ontogeny of the centriole and basal body, and particularly for answering the question of whether they contain DNA. It is with the isolation of the ULG chromosome by Hall *et al.* at Rockefeller University that these expectations have been brought to fruition.

Their strategy for the isolation of the



Pinwheel structure of basal bodies (ref. 11).

ULG chromosome took advantage of the extensive restriction fragment length polymorphisms (RFLPs) between the DNAs of *C. reinhardtii* and another *Chlamydomonas* species, *C. smithii*. A strain containing the *C. smithii* ULG and all other linkage groups of *C. reinhardtii* was created by crossing the appropriately marked strains. This was then subjected to repeated backcrosses to the *C. reinhardtii* parent. In each generation, markers on the ULG segregated together, whereas markers on the nuclear linkage groups segregated independently. This resulted in a convergence of the *C. reinhardtii* genetic background such that, by the seventh generation, genetic differences between siblings, such as RFLPs, have a high probability of being associated with the ULG.

By probing digested DNA from all four members of a tetrad from the seventh generation with a cloned genomic fragment containing a repetitive sequence, Luck's group was able to identify a single RFLP that showed the expected 2:2 segregation. This was used to screen a *C. reinhardtii* genomic library in bacteriophage lambda to isolate larger clones containing the putative *uni* DNA, the identity of which was established by correct segregation in the *C. reinhardtii*/*smithii* lineage.

That these cloned DNAs were indeed associated with basal bodies was dramatically confirmed by *in situ* hybridization. When cells were challenged with the *uni* probes, two bright headlights of fluorescence shone from the bases of the flagella. Hybridization required proteolysis of the cells, suggesting that the target molecule was buried within the structure of the basal body. Treatment with ribonuclease failed to affect the signals, confirming that they were derived from DNA and not RNA.

From its migration in transverse pulse-field gel electrophoresis and its resistance to degradation by γ -irradiation, the *uni* chromosome seems to be a linear molecule of 6–9 megabases. From quantitative hybridization experiments, it appears to be present in two copies per cell. These properties are consistent with the number

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of basal bodies in *Chlamydomonas*, but pose problems both for the well established haploid genetics of the *uni* genome and for the circularity of its genetic map. They also present a packaging problem in a cylindrical organelle having internal dimensions of 250×500 nm.

Nevertheless, these findings provide the first really convincing evidence for the existence of basal body DNA. The phenotype of the mutations encoded by the ULG strongly suggest that this DNA encodes structural components of the basal body, although clearly not all of them, as the tubulins that form the characteristic ninefold symmetrical structure are specified by nuclear genes⁹. As Hall *et al.* point out, structural complexity, specialized genetics, polyploidy and partial

dependence on nuclear genes for structural components are properties that the basal body shares with both mitochondria and chloroplasts. Like them, the basal body is thought to have a symbiotic origin¹⁰. It will be fascinating to see whether the ULG can be shown to have a homologue in prokaryotes, in mammalian centrosomes or in higher plants, which lack centrioles. The availability of a molecular probe for basal body DNA will not only provide long sought answers to the role of these fascinating structures in the cell, but may also give important clues as to the origin of the eukaryotic condition. □

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THERMODYNAMICS

What, if anything, is melting?

R. Bruinsma

MELTING is just about the most familiar of physical changes, so that it might be supposed that it is well understood. Remarkably, this is not so. Indeed, the thermodynamics of the transition continue to surprise. Calculations indicate that the melting of two-dimensional 'solids' is different from that of conventional three-dimensional solids. But a careful study by R. Geer and co-workers (*Phys. Rev. Lett.* **63**, 540–543; 1989) of melting in layers of carefully controlled thickness reveals that the real transition is quite unlike that predicted.

Conventional melting of the solid phase into the fluid phase requires the absorption of a certain amount of latent heat. Transformations between phases of a material during which latent heat is produced are 'first-order' phase transitions. Melting, for many years, was assumed to belong to this class. Fifteen years ago, J. M. Kosterlitz and D. J.

Thouless (*J. Phys. C* **6**, 1181–1203; 1973) suggested that matters could be rather different in two dimensions. Two-dimensional melting, they argued, should not involve any latent heat if the melting is triggered by the thermal production of dislocations, a type of structural defect. Subsequently, B. I. Halperin and D. R. Nelson (*Phys. Rev. Lett.* **41**, 121–124; 1978) showed that if this process prevails, then the melting transition is not directly from a fluid into a solid. Instead, a new intermediate phase — the hexatic phase — separates the solid and liquid phases, so that two phase transitions are encountered as the temperature is increased: from the solid to the hexatic and then to the fluid.

The hexatic phase should have both liquid-like and solid-like features. As a liquid, the hexatic cannot support shear stress. As a solid there are well defined crystallographic directions — six, in fact (hence its name). This phase turned out to be a elusive quarry. Computer simulations and X-ray diffraction experiments, on rare-gas monolayers absorbed on crystalline substrates, were controversial and inconclusive.

On the other hand, a hexatic phase was discovered, by R. Pindak *et al.* (*Phys. Rev. Lett.* **46**, 1135–1138; 1981), but near certain three-dimensional phase transitions. The materials studied were smectic liquid crystals, which consist of layers of rod-like organic molecules stacked on top of each other (Fig. 1a). Inside the layers, the molecules may either move freely as in a liquid (smectic A or Sm A) or they may be positionally ordered as in a crystal (Sm B). On heating, the Sm B phase normally transforms into a Sm A phase — just as for conventional melting. But, over a small temperature interval between the Sm B and Sm A phases, certain smectic liquid

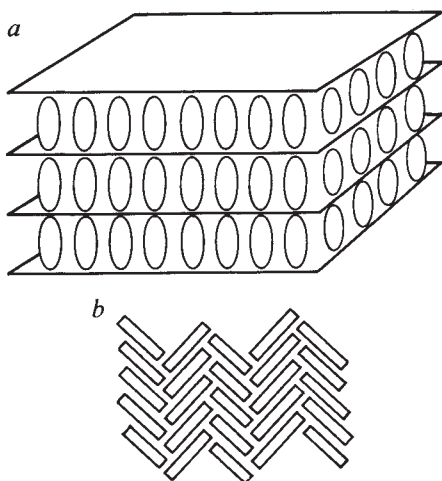


FIG. 1 Smectic liquid crystals: a, sideview; b, herringbone pattern (topview).

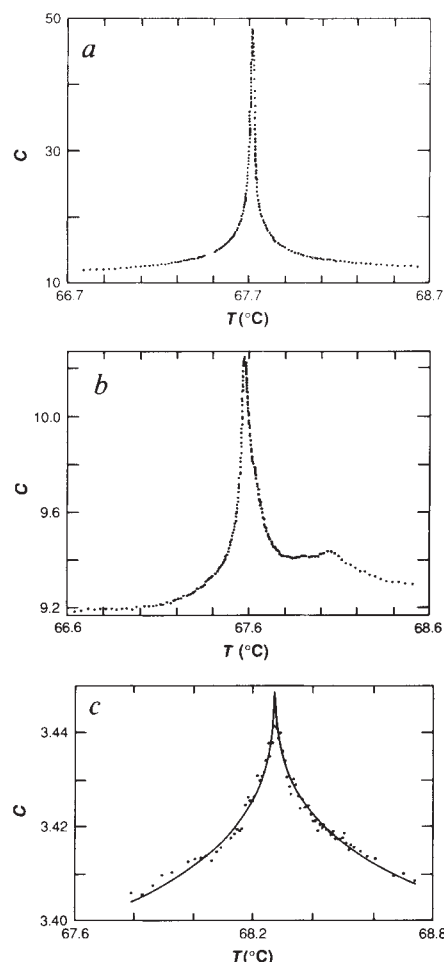


FIG. 2 Specific heat anomalies of 65 OBC liquid crystals (from Geer *et al.*). a, With 200 molecular layers, essentially three-dimensional, C diverges (goes to infinity) at the critical temperature; $C \propto (T - T_c)^{-\alpha}$, with α positive. b, With 10 molecular layers, a surface-ordering transition is apparent close to the bulk melting point. c, With just four layers, C reaches a maximum finite value at the melting point, and falls away as $C_p \propto -(T - T_c)^{-\alpha}$, with α negative on either side. For a first-order transition, the heat capacity would rise as a straight line (a δ -function) at the melting point, having no slopes on either side. It had been expected that the transition would have an 'essential' singularity, which should show only a broad maximum below T_c .

crystals, in particular the homologous series nm OBC (n and m are integers), have a third phase, Hex B, which has just the right orientational and positional correlations for a hexatic. The discovery of this phase immediately suggested a way of testing a theory of two-dimensional melting: make films of Hex B and watch what happens to the Sm A–Sm B transition as you lower the film thickness.

A possible diagnostic tool to investigate the nature of such phase transitions is the specific heat. For a first-order transition, the specific heat shows a δ -function singularity at the critical temperature T_c — that is, a very narrow peak. For a conventional second-order transition, the specific heat $C(T)$ diverges at the critical temper-

ature as $A/(T-T_c)^\alpha$, where A is the critical amplitude and α , the critical exponent. Also, the dislocations of the Kosterlitz–Thouless melting theory behave in a way similar to the ‘vortex’ defects of a plasmon ferromagnet (the so-called X – Y model). The specific heat of an X – Y model near its Curie (critical) point is known to have an ‘essential’ singularity at T_c , and the same is expected for the melting transition. In practice, such singularities are unobservable and $C(T)$ should be rather smooth, with a broad maximum somewhat below T_c . By measuring $C(T)$ we thus can tell which of the three cases applies.

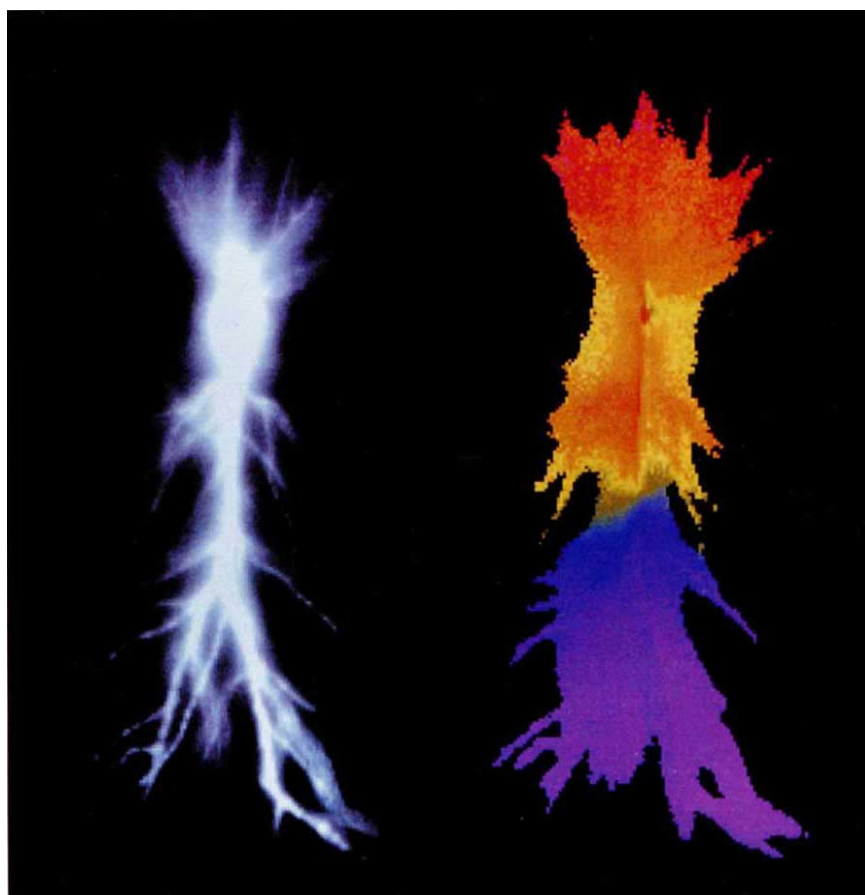
But measuring the specific heat of thin films is a demanding occupation. For films with a thickness of the order of monolayers, the specific heat of the sample holder is much greater than that of the film and fantastic precision is required. Nevertheless, Geer *et al.* have now succeeded in measuring $C(T)$ for very thin free-standing films of 65 OBC (Fig. 2). For temperatures near that of the bulk Hex B–Sm A transition, they found a single cusp-like specific heat anomaly — $C(T)$ rises to a finite maximum at T_c and does not diverge to infinity. The critical exponent α is thus negative; $\alpha \approx -0.26$.

This, obviously, disagrees both with the Kosterlitz–Thouless–Halperin–Nelson theory, which predicts no (measurable) singularity at T_c , and the conventional theory of first-order melting. We must, however, be cautious in drawing conclusions from their results. Real smectics are not just simply layers of ideal two-dimensional fluids of spherical atoms. The molecules carry internal structure. Furthermore, for smectic films, the molecules near the film’s surface behave rather differently from those in the interior.

To start with the internal structure problem, the ordered Sm B phase of bulk 65 OBC has an orientationally ordered herringbone-like pattern (smectic E) (Fig. 1b). Correlated clusters of molecules with local herringbone order are also clearly visible in the Hex B phase studied by Pindak *et al.*. In fact, it can be argued that the correlations, which assist orientational ordering, are instrumental in stabilizing the Hex B phase in the first place. At any rate, the interaction between the hexatic order and the herringbone fluctuations in bulk hexatics leads to so-called tricritical behaviour in the specific heat with $\alpha = 0.5$ — close to the observed critical behaviour for thick films of 65 OBC (R. Bruinsma & G. Aeppli *Phys. Rev. Lett.* **48**, 1625–1628; 1982). In two dimensions, this tricritical point should vanish (N. Berker, personal communication). This suppression of tricriticality on reducing the film thickness considerably complicates the analysis of the critical behaviour and may account for the vanishing of the divergence in $C(T)$ for thin films. (The authors appear not to account for this.)

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Calcium brightens up a brain cell



AFTER microinjecting individual cells in a brain slice with a calcium-sensitive fluorescent dye, David Tank and colleagues at AT&T Bell Laboratories are able to follow the accumulation of calcium in pyramidal cells of the guinea-pig hippocampus during synaptic activation. A fluorescent image of a pyramidal cell is shown on the left and a colour-coded map of its calcium content on the right (see page 433).

The second problem is the fact that the role of molecules right at the surface of the film becomes more important as we reduce the film thickness. For thicker films of 65 OBC one observes three phase transitions. Besides the Sm A–Hex B and Hex B–Sm B transitions, there is also a surface-ordering transition. On following these transitions with decreasing film thickness, Geer *et al.* find that for their thinnest films (four layers), the surface ordering transition and the Sm A–Hex B transition coincide. (It is not clear from the paper what happened to the Hex B–Sm B transition.) However, for film thickness greater than four layers, Geer *et al.* can still see the surface-ordering transition (Fig. 2b). The associated anomaly is, in fact, comparable in magnitude to the (presumed) Hex B–Sm A anomaly.

Unfortunately, this surface-ordering transition is at a temperature above that for the Hex B–Sm A transition, so it could not possibly be identified with the transition from the solid phase to the hexatic phase, which should be below the Hex B–Sm A transition. Apparently, a

completely separate surface phase transition is competing with the interior Sm A–Hex B transition. If the surface layer has Sm B positional order, hexatic order or herringbone orientational order, then this would change the interpretation of $C(T)$. At present, little is known about it. The physics of the molecules at the surface of the film is clearly rather different from those in the film interior, and for thin films we are left with a peculiar mix of surface and interior behaviour.

Despite these questions of interpretation, the demonstration by Geer *et al.* that the specific heats of such extremely thin films can be measured is a notable achievement. It could also be important in other fields where there is an interest in the critical behaviour of very thin layers, such as membranes, adsorbed monolayers or thin superconducting films. □

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Listening to the voice within

Alan D. Grinnell

LIKE most types of motor behaviour, vocalization depends critically on sensory feedback. Yet almost nothing is known of how or where this sensory-motor integration takes place in mammals. It is very satisfying, therefore, to see the report by Metzner on page 529 of this issue¹, based on extensive single-unit recording from awake, spontaneously-vocalizing mammals, that identifies a site and provides a cellular mechanism for auditory feedback control of one of the most precisely regulated vocalization behaviours known: Doppler-shift compensation in echolocating bats.

Several species of insectivorous bats use echo-location sounds composed of a 10–100-ms-long constant-frequency component, terminating in a brief, downward frequency sweep. The frequency of the constant component (approximately 78 kHz in the rufous horseshoe bats, *Rhinolophus rouxi*, used in this study) is remarkably accurately regulated, to less than ± 50 Hz, for sounds emitted by a particular bat at rest. Echoes of sounds emitted in flight return Doppler-shifted by up to 4–5 kHz, depending on the relative velocity between the bat and target. Bats use the small frequency and intensity fluctuations in the echo, due for example to insect wing movements, to detect and identify targets².

Two remarkable and well-documented adaptations make this possible. First, the auditory nervous system is sharply tuned to a frequency very close to that of the constant frequency at rest, so that small changes in echo frequency cause large changes in response^{3,4}. Second, to take advantage of this sharp tuning, the bat lowers the frequency of its next emission just enough to compensate for the upward Doppler-shift in the most recent echo, so that the echo is always held close to the region of maximum auditory sensitivity⁵. This Doppler-shift compensation can be as accurate as ± 50 –100 Hz, representing a sensory-motor regulation to within about 0.1 per cent of the constant frequency, over a frequency range of 5 per cent or more. Few, if any, more accurate forms of sensory-feedback regulation exist.

Previous research has yielded clues to where one might look for auditory feedback control of vocalization. The constant-frequency component of the emitted sound is controlled by the degree of contraction of the cricothyroid muscle; this, in turn, is directly correlated with the

discharge rate of the superior laryngeal nerve arising in the nucleus ambiguus in the brainstem⁶. Ablation experiments indicate that the behaviour of Doppler-shift compensation can be controlled at the midbrain and tegmental levels^{7,8}, and there have been reports of attenuation of evoked or single-unit responses in the midbrain and cortical levels during vocalization in bats and primates^{9,10}.

Most interesting is Schuller's description¹¹ of four units in the inferior colliculus of *R. ferrum-equinum* that respond differently or respond only to a sound when it is coupled to an electrically elicited vocalization. None of these studies, however, reveals behaviour that helps explain their

is strongly inhibited during each vocalization (hence the name VOC-inhibitory units). But when a copy of the emitted sound, reduced in intensity, delayed in time, and shifted in frequency to simulate an echo, is replayed to the bat overlapping its vocalization, all of these units show phasic-tonic responses to the 'echo'. This is remarkable behaviour: the units fail to respond to the bat's outgoing sound and their background activity is inhibited during the vocalization, yet they respond well to a fainter, overlapping, Doppler-shifted echo. The requirements of 'echo' delay and frequency differ for different units, with each showing a maximum response to a given delay and shift in frequency. Significantly, the ranges of delay and frequency shift are precisely those that are effective in eliciting behavioural Doppler-shift compensation.

The pattern of frequency sensitivity of these VOC-inhibitory neurons is particularly important. They respond with exquisite sensitivity to changes in frequency of 100 Hz or less, going from 10 to 100 per cent of maximal response with a change of only about 1.5 kHz, and then maintain maximal firing rates with a further increase of 2–3 kHz. This is quite unlike most other units studied, either auditory or audio-vocal, in that the frequency-response curves fall off steeply on the low-frequency side and relatively slowly on the high-frequency side. This is critical to their postulated role in mediating Doppler-shift compensation.

Horseradish peroxidase injection studies show several indirect connections between the PLZ and the nucleus ambiguus, and a high proportion of PLZ neurons express glutamic acid decarboxylase, suggesting that their neurotransmitter is GABA. Given these connections and the properties of VOC-inhibitory units, it is possible to build a model that can explain Doppler-shift compensation. An echo with a slight upward Doppler-shift excites some of the VOC-inhibitory neurons, which send inhibitory input to the nucleus ambiguus, reducing the firing rate of superior laryngeal motoneurons. With larger Doppler-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Rhinolophus rouxi — flying blind.

sensory-feedback control of vocalization.

Metzner¹ has now surveyed single-unit behaviour in much of the midbrain and brainstem in freely vocalizing bats. Of greatest interest is a restricted tegmental region rostral and medial to the nuclei of the lateral lemniscus, corresponding to the paralemniscal zone (PLZ) of other mammals. Not only are most PLZ units responsive to acoustic stimuli, but a majority also show firing patterns correlated with vocalizations (but not respiration). Two types of units fire in bursts before each vocalization, with the firing rates inversely correlated either with the duration or the frequency of the constant-frequency component.

Most interesting, however, is a third type, constituting approximately half of the audio-vocal units in the PLZ. These exhibit properties that make them ideal candidates for mediators of Doppler-shift compensation. Their spontaneous activity

Alfred Limbrunner

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shifts, because of the broad high-frequency sensitivity of the units, more of the population fires maximally, increasing the inhibitory input to the motor nucleus and further reducing the constant frequency in the next vocalization. The source of the delay selectivity of VOC-inhibitory neurons is not known, nor is the nature of the excitatory input that can overcome the inhibition during vocalization. Metzner postulates the need for convergence of an excitatory response to the echo with an excitatory input triggered at variable delays by the vocalization.

There are other details to be filled in, of course. But this is a rich story and a significant advance in our understanding of the sensory-motor interactions responsible not only for the regulation of this most remarkable behavioural phenomenon but for audio-vocal control in mammals in general. Bats, with their overwhelming dependence on audition and their use of simple, stereotyped signals, have taught us much of what we know about information processing in the auditory nervous system; they are now doing the same for audio-vocal integration. □

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deposited energy, explosion is divided from collapse. What is worse, calculations claimed successfully to predict explosions have frequently relied on a strong outgoing shock — that is, one travelling at more than the escape velocity. This particular condition has always seemed to me to be unnecessary and insufficient. It is the peculiar phenomenon of a hot bubble in Wilson and Mayle's new model that circumvents this arcane dependence. It is because of the natural formation of a hot, very high entropy bubble surrounding the neutron star that persistently pushes out the ejecta that I believe this long and difficult problem is solved.

A strong shock wave in matter is the signature that something is pushing with enough force to ensure that the resultant velocity of the matter is greater than its initial sound speed. The shock itself is merely the singularity associated with the inelastic collision that occurs when stationary matter is converted (shocked) to moving matter. Because an inelastic collision always converts the relative kinetic energy to heat, it is evident that the increase in internal energy of shocked matter corresponds to its change in kinetic energy. And because of this internal energy, the flow immediately behind a strong shock is subsonic; that is, a sound wave produced by a perturbation behind a shock will always catch up with the shock. This is fortunate because, if it were not true, a strong shock, once launched, would go on forever because it would not 'know' that its driving pressure had

Hot bubbles drive explosions

Stirling A. Colgate

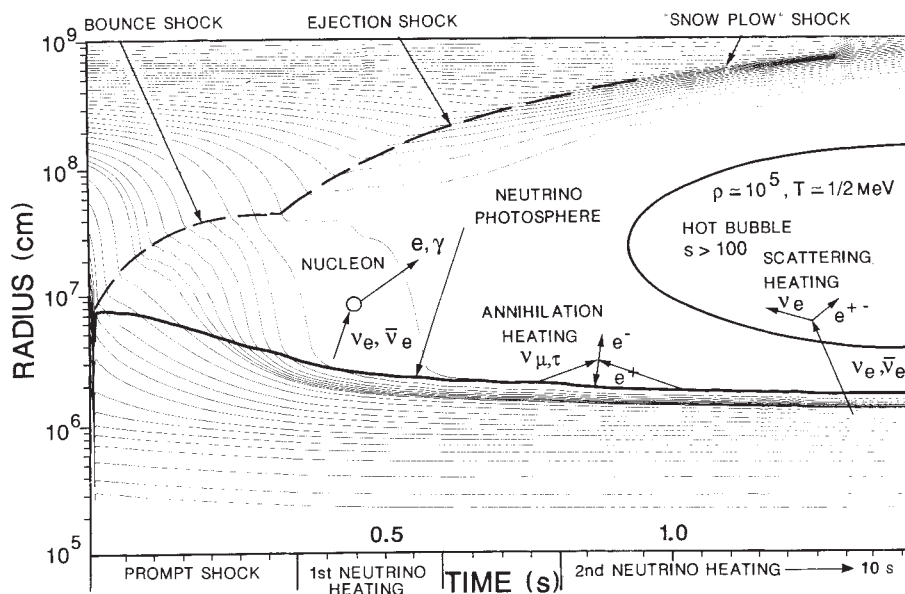
OUR understanding of the initial moments of a supernova was convincingly confirmed by the events following SN1987A: the exhausted core of a main-sequence star collapses under its own weight and emits a massive pulse of neutrinos. Several hours later the explosion of a highly evolved, 12-solar-mass star is observed. The computer modelling of the collapse and emission of the gravitational binding energy of the core in a massive pulse of neutrinos was marvellously confirmed. Similarly, computer modelling of the 'generic' explosion produced, in this case, remarkable agreement with the details of the observed light curve, X-ray, γ -ray and infrared emission in a whole range of details. But the excitement generated by this extraordinary agreement suppressed one leading difficulty: why does the star blow up instead of collapsing to a black hole? New calculations by James Wilson and Ron Mayle¹ (Lawrence-Livermore National Laboratory) presented at a recent meeting* give the first convincing answer to this question.

Our planet and life exist only because of the ejection of high-mass nuclei synthesized in supernovae, which is why the explosion mechanism is of such interest. The gravitational binding energy released in the initial burst of neutrinos that accompanies the collapse (3×10^{53} ergs) exceeds the final kinetic energy of the ejecta (10^{51} ergs = 1 'foe') by a factor of 300. Therefore, in a computational model, one has to couple a mere 0.6 per cent of this binding energy to the ejected matter (half of which is used in overcoming the initial gravitational binding of the ejected matter) to generate an explosion.

This is what we² first attempted some 30 years ago at Lawrence-Livermore using computer codes from the nuclear-weapons programme with gravity and neutrino

scattering cross-sections added. Subsequent generations of researchers (including many leading astrophysicists) have used models of ever greater complexity, adding refinements and new physics (particularly neutral-current neutrino scattering) to search for a mechanism that convincingly results in an explosion, with 1 foe, rather than a collapse. Wilson and Mayle have finally demonstrated such a model in which a hot, high-entropy bubble produced by neutrino-antineutrino annihilation³ pushes the ejecta.

Countless calculations teeter at this 0.6-per-cent edge, at which fraction of



The sequence of processes occurring in a type II supernova according to Wilson and Mayle. First a star collapses, owing to exhaustion of the fuel and emits electron neutrinos as the compressing matter turns into neutrons. The forming neutron star bounces producing a strong shock that weakens owing both to the thermal decomposition of nuclear matter and the reversal of the neutron star bounce trajectory. Subsequently, a large burst of neutrinos of all flavours heats the infalling matter, starting an explosive shock. Yet later, mu and tau neutrino-antineutrino annihilations in nearly opposed collisions just above the neutrino photosphere generate heat without nucleonic matter, making a hot, high-entropy (100 Boltzmann-unit) bubble, which pushes out the ejected matter in a near 'snow plow', that is, a thin shock. The pressure is maintained because the high-entropy ensures a large scale height adjacent to the neutron star.

* Tenth Santa Cruz Summer Workshop, *Supernovae*, 10–21 July 1989.

disappeared; the Universe would now be filled with strong shocks — an awkward place in which to live. Instead, the driving pressure disappears, and the resulting rarefaction soon reaches the shock, which weakens, which is the failing of previous supernova models.

When the inner regions of a star first collapse, producing a neutron star, there are two mechanisms that give a strong shock in the matter still falling inwards. The first is the elastic bounce of the dynamically forming neutron star, the second is the heat produced by the interaction of a small fraction of the neutrinos that are emitted from the forming neutron star. The energy residing in mechanical motion of the forming neutron star is relatively small, perhaps a few foe, but it is coupled efficiently to the external matter. The neutrino flux carries 100 times more energy, but neutrinos are only weakly coupled to the external matter because of their small scattering cross-section. Hence, many calculations produce a strong shock that barely exceeds (or is sometimes less than) the escape velocity; success or failure is announced accordingly.

The problem is twofold. First, the calculations are terminated after, at best, several seconds because the computer budget is exceeded. Second, the analytic extrapolation neglects the almost certain formation of a rarefaction wave emanating from the cooling neutron star that will reach the shock, reverse the flow and produce a black hole rather than an explosion. Initially, the neutron star formed is so hot that the neutrino flux heats all the matter adjacent to itself and initiates an explosion. But after roughly 10 seconds — the duration of the neutrino pulses measured at the Kamiokande and Cleveland detectors — the neutron star has cooled so that matter next to it is captured and compressed by the extreme gravity; what is more, any of the matter's internal heat, previously causing the pressure that drove the explosive shock, is now itself cooled and thus the matter collapses back onto the neutron star.

The cooling, which arises from electron collisions of the form $e^- + e^+ \rightarrow \nu_e + \bar{\nu}_e$, occurs in about $200 T^{-5}$ seconds, where T is the temperature measured in megaelectron volts (MeV). One MeV, equivalent to 10^{10} kelvin, may not seem cool, but close to a neutron star (where the extreme gravity gives a potential energy of 100 MeV per nucleon) it is sufficient to raise normal matter (with a comparable number of nucleons, electrons and photons) to a scale height only 1 per cent of the neutron star's radius. So the push from such matter above 100 scale heights (double the neutron-star radius), where the pressure and density are smaller by e^{-100} , is negligible.

The result of this cooling is that the pressure driving the explosion shock would disappear after about 10 seconds.

Without some continuing pressure most of the matter previously shocked out to radii of 10^{10} cm would fall back onto the neutron star. With only a small fraction of this matter, the neutron star would collapse further to produce a black hole. But we find neutron stars in at least half of type-II supernova remnants and the events that followed SN1987A would have been vastly different if a black hole were at its centre.

According to Wilson and Mayle, what circumvents this second collapse is the extraordinary process of neutrino–antineutrino annihilation³, mostly between mu and tau neutrinos, immediately adjacent to the neutron-star surface roughly half a second after the initial collapse (see figure). Neutrino annihilations produce energetic 20–30-MeV electrons and positrons that are immediately thermalized. The mu and tau neutrinos, having smaller cross-sections for scattering by nucleons, emerge from deeper within the neutron star and so are hotter, $T \approx 10$ MeV, and create a larger flux than electron neutrinos. Head-on collisions are needed, which in turn requires that the density gradient at the neutron star's surface is steep: neutrinos at a steep surface are emitted isotropically, whereas at a shallow surface, they tend to stream radially outwards. The result is heating regardless of nucleon content so that a region of very high entropy is formed.

The entropy is a measure of the ratio of the number of electrons and photons (light particles) to nucleons (heavy particles). If the entropy is high, greater than 100, the corresponding scale height of the atmosphere around the neutron star becomes greater than the star's radius so this atmosphere can push efficiently against the ejected matter and keep the shock going. The hot bubble in Wilson and Mayle's calculation reaches entropies as large as 10^4 , so that the pressure is assured provided the bubble does not cool next to the neutron star. This is avoided in the calculation because the temperature required to sustain the pressure is less than that of the cooling neutron star.

This extraordinary process of collapse, bounce, first neutrino push, mu or tau neutrino–antineutrino annihilation, hot bubble formation, and finally explosion would be impossible to believe without the evidence from SN1987A. The model would surely be confirmed if it is shown to be capable of predicting the mixing of ^{58}Ni from the inner atmosphere into the atmosphere as observed at SN1987A. □

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Quick reactions

MICROWAVE heating is bringing about a small revolution in chemistry. All sorts of reactions go much faster in a microwave oven than over a traditional burner, and it is hard to believe that the effect is merely thermal. Daedalus points out that many molecules, and most reaction intermediates, have a dipole moment: in the oscillating microwave field, they will spin. If in addition they are even slightly chiral, this spin will drive them along hydrodynamically like little propellers. Even a small directional effect like this should bring reacting molecules together, and drive the products apart, far faster than mere random-walk thermal diffusion. No wonder reaction rates go up.

But, says Daedalus, this effect as it stands is still very inefficient. At any instant, a simple oscillating field will spin a polar molecule clockwise or anticlockwise with equal probability. If it is any sort of propeller, it will shuttle rapidly back and forth along the field-rotation axis; moving much faster than by thermal diffusion, but still in a wasteful one-dimensional random walk. What is needed is a pure rotating field, to spin the molecules one way only. Asymmetric polar molecules would then be powered around a reaction mixture with unprecedented speed and force. DREADCO's circularly polarized microwave oven (available in clockwise or anticlockwise versions) should have a stunning effect on reaction rates, even at settings so low that its pure heating action is quite negligible.

Even more intriguing, it may enhance chiral reactions. Claims have already been made that a reaction mixture spun clockwise produces a different yield of *dextro* and *laevo* enantiomers than one spun anticlockwise; and electrically directed molecular spin should be far more effective than mere bulk angular momentum. Irradiated with circularly polarized microwaves, a chiral reaction should give just one of its possible asymmetric products, with little or no contamination by molecules of opposite handedness.

Biochemistry in particular will benefit. For most of the molecules of life are both polar and chiral; their reactions should be wonderfully enhanced by circularly polarized microwaves. Already DREADCO's biochemists are irradiating bacterial and fungal cultures of all sorts, looking for enhanced growth rates, rapid acceleration of metabolic cycles, improved yields of chirally pure antibiotics, and so on. Conversely, by reversing the sense of the irradiation, the crucial reactions of life could be choked off, or gummed up with useless molecules of the wrong handedness. Daedalus may have invented a new food-irradiation technique, free of the worries invoked by γ -ray treatment. David Jones

Ubiquitin in a togavirus

SIR—Khatchikian *et al.*¹ have shown that insertion of a 28S ribosomal sequence into the haemagglutinin gene of an influenza virus leads to increased viral pathogenicity. We now report the identification in another RNA virus of inserted cellular sequences, which may also be linked to the pathogenesis of a viral disease.

Pestiviruses, currently classified in the family Togaviridae, are positive-stranded RNA viruses that cause severe animal epidemics including bovine viral diarrhoea and hog cholera. Propagation of the bovine viral diarrhoea virus (BVDV) in tissue culture allows one to discriminate between cytopathic (cp-BVDV) and non-cytopathic (noncp-BVDV) strains, both of which can be pathogenic in cattle. One remarkable difference between the two strains is that a non-structural virus-encoded protein (p120) of unknown function is processed to a small product of *M_r* about 80,000 only in cp-BVDV-infected cells. Interestingly, the coexistence of both biotypes in persistently infected animals is linked to pathogenesis of fatal mucosal disease².

On comparing the genomic sequences of two cp-BVDV strains Osloss³ and NADL⁴ and one hog cholera virus strain⁵ we found insertions in the BVDV gene

with different strains of cp-BVDV. The ubiquitin probe recognized genomic RNA from the Osloss strain only (see figure; the other three bands visible in all lanes obviously represent bovine ubiquitin messenger RNAs).

Based on these observations, we would like to suggest a novel model for the pathogenesis of mucosal disease. In persistently infected animals a noncp-BVDV mutates to a cp-BVDV biotype by taking up cellular sequences during a recombination event. This would explain the isolation of serologically closely related noncp-BVDV and cp-BVDV from one animal suffering from the disease^{6,7}. Once this recombination has occurred, horizontal transmission of the resulting cp-BVDV strain follows. Further efforts

will be directed towards a comparison of cp-BVDV and noncp-BVDV strains, including the detailed investigation of their p120 and its processing.

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Particle migration on cells

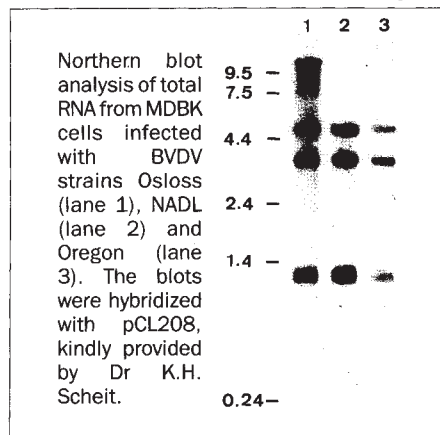
SIR—In their recent article entitled 'Nanometre-level analysis demonstrates that lipid flow does not drive membrane glycoprotein movements', Sheetz and colleagues claim to have eliminated the membrane flow hypothesis of cell locomotion². Their conclusion arises from experiments in which they add 40-nm gold particles coated with concanavalin A (Con A) to migrating macrophages. Using an ingenious optical technique involving image processing, they follow the movements of individual gold particles on the dorsal surfaces of these cells. They observe that the particles alternate between two modes of behaviour: either they diffuse by brownian motion, or they move (more or less directly) away from the leading edge of the cell. As analysis of particles in the diffusive mode shows that there is no superimposed directional drift on a particle, they believe that there can be no bulk (lipid) flow in the cells' plasma membranes. They suggest that the alternative mode — direct motion away from the leading edge — is driven by the actin cytoskeleton.

There appears, however, to be a conceptual error in the article. In measuring the diffusion of particles on the dorsal surface (finding there is no superimposed drift), the authors use a stationary marker on the glass slide as a reference point (see legend to Fig. 2 in ref. 1). But if the cells are actually moving (which is assumed, but not shown), then any particle on their dorsal surface that is stationary with respect to the substratum must be moving rearwards with respect to the cell. In other words, the particles, even when in a diffusive mode, must be moving away from the cell's leading edge. This is what is expected

on the membrane flow scheme (see ref. 3, where this point has been discussed); the observations of Sheetz *et al.* could therefore be used to argue that membrane flow does exist in these cells.

There is an additional problem in the interpretation of the observations. Sheetz *et al.* assume the particles are on the cells' dorsal surfaces, but do not establish this. They could be inside the cells, or between the cells and substrate (under the cells). The reason for this is that the thickness of the leading lamella of a cell is usually about 250 nm (for example, ref. 4 shows the lamella varies in thickness between about 150 nm to 700 nm in one fibroblast), and the depth of focus of the microscope used is 250 nm (M. Sheetz, personal communication). It seems likely that some of the particles observed are inside the cells because macrophages are very active in endocytosis and phagocytosis. Con A actually stimulates membrane internalization in these cells⁵, about half the surface-bound Con A being internalized in a few minutes⁶. Alternatively, it is conceivable that the particles are semi-trapped under the cells. In either case, if the particles are not on the dorsal surface, conclusions derived from the assumption that they are, would not be valid.

Finally, Sheetz *et al.* resurrect one of the old arguments against membrane flow: the intracellular vesicles that, on the membrane flow scheme, are supposed to move forward inside the cell and exocytose at the cell's leading edge have



coding for p120, which may affect its processing. The Osloss insertion comprises 228 nucleotides, the NADL insertion 270 nucleotides. The two inserted sequences are not similar in either nucleotide or deduced amino-acid sequence. Whereas the NADL insertion is not similar to any sequence in the current data bank, the amino-acid sequence deduced from the Osloss insertion is almost identical to animal ubiquitin: compared to the ubiquitin sequence conserved in all animals⁸, only 2 of the 76 amino acids differ.

To examine whether the ubiquitin coding sequence is specific for the Osloss strain, a *Bgl*II fragment derived from a porcine polyubiquitin complementary DNA clone (pCL208, ref. 7) was hybridized to total RNA of cells infected

1. Sheetz, M.P., Turney, S., Qian, H. & Elson, E.L. *Nature* **340**, 284–288 (1989).
2. Bretscher, M.S. *Nature* **260**, 21–23 (1976); *Science* **224**, 681–686 (1984).
3. Bretscher, M.S. *J. Cell Biol.* **106**, 235–237 (1988).
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5. Edelson, P.J. & Cohn, Z.A. *J. exp. Med.* **140**, 1364 (1974).
6. Berlin, R.D., Oliver, J.M., Walter, R.J. *Cell* **15**, 327 (1978).
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not been 'seen', although there is no obvious reason why they should be visible with current techniques. In an accompanying letter⁷, Sheetz and colleagues report that their Con A labelled particles "on" fish keratocytes sometimes move long distances forward at great speed. Could it be that they have discovered just those *intracellular* vesicles they claim do not exist?

In the light of all this, the accompanying and enthusiastic obituary of the "lipid-flow model" in *New and Views*⁸ seems premature.

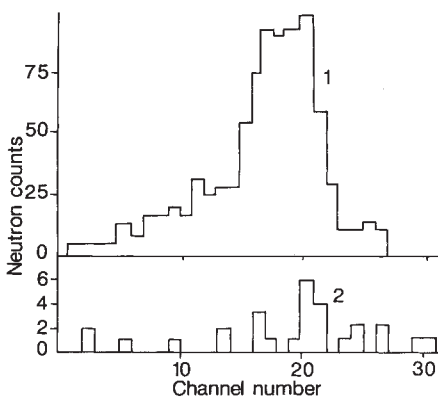
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Titanium fracture yields neutrons?

SIR—In recent experiments^{1,2}, we showed that violent mechanical action on heavy ice and lithium deuteride lead to neutron emission at levels substantially above the background, indicating the occurrence of d-d nuclear reactions. Because of current interest in the possibility of d-d reactions as a result of saturating titanium and palladium with deuterium^{3,4}, we felt that a test of mechanical agitation of titanium in the presence of deuterium was warranted.

Titanium chips of technical purity were tested in conjunction with heavy water, deuterated polypropylene (PP) and lithium deuteride. Ti chips were put in a vibromill, operated at 50 Hz and with an amplitude of 5 mm (the power applied was $\sim 10 \text{ Wg}^{-1}$). Two-thirds of the volume of the vibromill drum was filled with steel



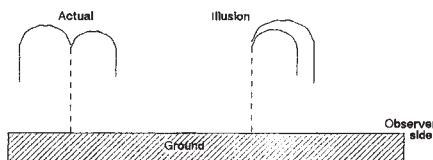
Histogram of neutron counts versus analyser channel for a calibrated neutron source (curve 1) and for Ti chips agitated with 10% D₂O and 4% PP(D₆) (curve 2).

balls 6 mm in diameter^{5,6}, the remaining one-third comprising Ti chips and deuterated material. In control experiments, finely crushed Ti chips were used separately, as well as D₂O and PP(D₆).

Our neutron detector was a block of seven proportional counters, immersed in a tank of oil and covered with cadmium

Pyrotechnic illusion

SIR—While watching a recent display of fireworks, I observed a robust illusion of motion. Clearly, when a flare shot into the sky bursts, and its payload of self-luminous fragments scatters, there must be a uniform distribution of horizontal directions of fragment trajectories, with respect to the vertical axis formed by the initial shot. However, all fragments appear to come towards the observer, or at least into the hemisphere formed around the observer and centred on the flare's explosion point. Even when one is consciously aware of the illusion, it seems impossible to decide which fragments are



really going away. Apparently trajectories going away are so similar to trajectories coming towards the observer that the brain interprets all motion parsimoniously as movement towards the observer. It is important that the background is black,

with respect to the bright, moving fragments; fireworks seen against clouds or against textured stadium backgrounds can be analysed for towards/away fragment motion. Even when viewed through the limited field of binoculars, the initial burst of fragments retains the illusion of motion only towards the observer.

Of course, after a second or so all horizontal motion of the fragments ceases and only earthward motion is seen. However, there are special fireworks with fragments which change direction after the initial burst, and some of these fragments can be seen going momentarily away from the observer. Pyrotechnics seen on television show the same illusion, but less dramatically than those seen in person.

I have found no mention of this phenomenon in books. Colleagues have suggested various explanations, my favourite being the notion that the initial burst is rapidly enlarging and an enlarging object is interpreted as coming towards the observer (S. Ullman, personal communication). I wonder if other readers of *Nature* can offer more insights.

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sheet, placed 15 cm from the vibromill drum. Detector counts were brought out to an AI-256-6 analyser. Detector efficiency was measured using a source of 200 neutrons s⁻¹ intensity placed in the vibromill drum (curve 1 in the figure), and at regular intervals the neutron background was measured by removing the operating drum to a large distance.

In our experiments, neither Ti chips nor deuterium containing compounds showed a neutron signal above background (0.05 counts s⁻¹) when put separately in the drum, but when Ti chips were combined with 10% heavy water or 4-5% PP(D₆), or with both, a neutron flux 6-7 times background ($0.31 \pm 0.13 \text{ counts s}^{-1}$, taking detector efficiency into account) was measured, as indicated in curve 2 in the figure, which shows the results from Ti + 10% D₂O + 4% PP(D₆). A constant excess over background was measured for 10 minutes, with a subsequent attenuation after crushing of the chips was stopped. The greatest effect ($0.41 \pm 0.14 \text{ counts s}^{-1}$) was measured using 10% D₂O and 4% PP(D₆) when the vibromill drum was cooled in liquid nitrogen after mechanical action was stopped. After three or four cycles of vibration, each lasting 3 minutes, neutron emission fell to the background level. Smaller effects ($0.14 \pm 0.02 \text{ counts s}^{-1}$) were found using LiD with PP(D₆).

We conclude that d-d reactions occur when Ti particles in the drum are saturated with deuterium, perhaps as a result

of deuterium diffusion through freshly created Ti surfaces produced by the fracturing⁶. At least 2 atoms of D per atom of Ti can be absorbed, and the lattice of Ti particles deforms by as much as 25% during this absorption⁷. So it is conceivable that absorbed deuterons can approach each other closely enough for fusion to occur. In addition, electric fields of up to 10^7 V cm^{-1} can be created by destruction of the crystal lattice, perhaps making d-d reactions more probable⁸. The increase of neutron counts with cooling in liquid nitrogen may indicate increased absorption of D, as it is known that cooling decreases the partial pressure of absorbed hydrogen or deuterium⁷.

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The neuroanatomist

Sanford L. Palay

Cajal on the Cerebral Cortex: An Annotated Translation of the Complete Writings. Edited by Javier DeFelipe and Edward G. Jones. Oxford University Press: 1989. Pp. 654. £50, \$65.

THE last quarter of the nineteenth century was marked by a steady increase in knowledge about the nervous system. Advances in neuroanatomy, neurophysiology, psychology, psychiatry, clinical neurology and pharmacology arrived in rapid succession, so that by the beginning of the twentieth century a remarkable body of knowledge had accumulated. Here was a storehouse of questions and problems that are only now giving up their answers to new and more powerful techniques.

Naturally, progress on all fronts was not even, and during the last decade of the century neuroanatomy advanced more rapidly and more consistently than any of the other disciplines. That phenomenal growth was due almost entirely to the work of one man, Santiago Ramón y Cajal, who from 1888 to 1911 almost single-handedly created modern neuroanatomy. The centenary of Cajal's birth in 1952 passed virtually unnoticed, but the 130th anniversary was marked by the publication of a splendid book of photographs of Cajal's life and a text in Spanish by P.L. Entralgo and A. Albaracín. The fiftieth anniversary of his death in 1984 provided an opportunity for commemorating the life of this remarkable scientist and opened a spate of celebrations of his creative activity. A symposium was held at that time in Valencia. Last year a symposium was held in Barcelona to honour the centennial of his first publication, in 1888, on the structure of the nervous system, his analysis of the architecture and development of the cerebellar cortex. A few months later the book reviewed here was published by Oxford University Press. Just recently, MIT Press has reissued Craigie's English translation of Cajal's autobiography, which had been out of print for almost two decades. These centennial anniversaries prompt a return to the original texts in order to re-evaluate their contribution to modern neuroscience.

Cajal on the Cerebral Cortex consists of English translations, set in chronological order, of all Cajal's works on the structure

and organization of the cerebral cortex. The text is divided into four periods — early, middle, consolidation and final — and each section is introduced by a summary that places the text in its time both in Cajal's life and *oeuvre* and in



Layer of the tasselled pyramids — a drawing by Cajal of the olfactory sphenoidal cortex from a one-month-old child. (Taken from *Cajal on the Cerebral Cortex: An Annotated Translation of the Complete Writings*.)

the science of the period.

The book is rounded off by an interesting chapter of more than 60 pages describing and analysing Cajal's materials, his methods of work and drawing, as well as the importance of his specific contributions to cortical histology. DeFelipe and Jones examined Cajal's own preparations preserved in the archives of the Cajal Institute in Madrid, and even found the same cells that he drew, so that they could photograph them. This chapter compares Cajal's work with modern investigations. It shows how prescient he really was and how closely modern neurohistology has

followed and corroborated his diagrams and interpretations (which is not to say that he did not make any mistakes or that nothing new has been added to his work). This chapter is a small jewel, expertly assimilating the details of Cajal's voluminous study of the cortex into the most recent morphological, cytochemical and connectational investigations.

The translators have chosen to follow the language of the original texts as closely as possible in order to render into English "the cadences and . . . color of Cajal's prose". Unlike certain earlier translators, they do not paraphrase his thoughts, but give an equivalent expression in English as far as possible. They have had to do more than translate individual words and phrases, however, for they have had to modernize many terms in order to make them comprehensible to a present-day audience. Nevertheless, they could not resist the temptation to translate Cajal's *etranglements* into *strangulations*, no doubt picturesque, but hardly comprehensible to the neuroscience graduate-student who has never seen a live node of Ranvier. Despite a few rough passages, the text is generally in graceful English and does credit to Cajal's style as well as to the scientific content.

The translation itself begins with Cajal's first paper on the texture of the cerebral gyri of the lower mammals from the *Gaceta Medica Catalana* of December 1890, and it reaches a climax with the great chapters on the cortex in the *Histologie du Système Nerveux*. In the first paper, Cajal describes the cells of the molecular layer of the cortex and the nerve fibres in this layer that originate from the cells of Martinotti in underlying layers. This is a topic to which Cajal often returned, correcting his early mistakes and enlarging his description. In fact, the succeeding papers, lectures and book chapters give the student a chance to follow the development of an astute, careful and absolutely honest observer as he increases his skills, learns from his mistakes, enlarges his understanding. This is a rare opportunity. There are few scientists whose intellectual development can be so carefully followed and whose development can be traced so rewardingly. For Cajal was always looking for the principles in the detail. He tried to describe and draw what he saw as exactly as possible; but he never lost sight of his aim, which was to understand how the nervous system was constructed so that its function could be discerned. ►

The translators have performed a signal service in scholarship by comparing the Spanish and French and, where necessary, the German versions of the same texts that were published in Cajal's lifetime. Footnotes at the end of each paper explain where the texts differ, where Cajal added something in one language that was not present in the original, or corrected a mistake in the original by further research before the non-Spanish version was published. These inklings into the mind of the master make the footnotes as interesting as the texts.

The translators have also re-created almost all of Cajal's references. Although they say that they had "little difficulty in identifying the vast majority of the works to which he referred", it could not have been an easy task to reconstruct full titles and journal references from Cajal's often sketchy citations. These are given not only at the end of each translated paper, but also in alphabetical order as a bibliography at the end of the volume. A useful index completes the book.

During his early career, Cajal felt isolated because his contemporary scientists did not read Spanish and ignored him. But even in those days, the best scientists knew several European languages and travelled widely. Koelliker carried on a long correspondence in Italian with Golgi,

and he learned Spanish in order to read Cajal's works in the original. Besides, there was a strong interest in translating important works into the most active scientific languages. Golgi was translated into German and French, and so was Cajal. But in the late twentieth century all scientists are expected to understand English, and the classic works that everyone in earlier times knew and could read in the original now go unread and unknown by most neuroscientists, simply because they find it too much trouble to read another language. Few of our contemporaries have read his work, and for these the principal source is his great book, the *Histologie du Système Nerveux de l'Homme et des Vertébrés*, translated into French from the Spanish and much enlarged.

Parts of Cajal's works had been translated into English before (those on the retina, the hippocampus, the regeneration of nerves, the embryology of the nervous system and the neuron theory). The present volume adds an extremely valuable set of papers to these earlier translations. It makes them available to a new generation of neuroscientists, who can find their roots in the work of genius of a century ago. □

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Cajal by himself

SANTIAGO Ramón y Cajal's autobiography *Recuerdos de Mi Vida* was published in instalments between 1901 and 1917, the period when he reaped his rewards for 20 years of passionate devotion to describing the structure of nervous systems. The English translation by E. Horne Craigie, *Recollections of My Life*, appeared in 1937. As Sanford Palay points out above, it has recently been reissued in paperback by MIT Press with a new foreword by W. Maxwell Cowan. This is a delightful account of the development and flowering of one of the most creative individuals in modern biology.

The son of a poor country doctor in the hill country of north-east Spain, Cajal was a rebellious, ingenious child with considerable artistic and musical talents but little love of book learning. He scraped through medical training and it was only after a serious illness, contracted on military service in Cuba, and an early marriage, that his imagination was captured by descriptive and microscopic anatomy. Poverty deterred him from the then-fashionable pursuit of microbiology, and an encounter with Camillo Golgi's new method for staining nerve cells convinced him that his life's work lay in studying the nervous system.

By his own account an obsessive, Cajal also had a breadth of interests from politics to chess and photography. The last of them contributed significantly to his career as a

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Cajal in 1934 at the age of 82.

neuroanatomist: his experience with manufacturing gelatine-bromide plates introduced him to the chemistry of silver salts, the basis of many stains for nerve cells, including Golgi's. He also invented a photolithographical technique for reproducing his splendid drawings of neurons.

Working in an isolated academic community, Cajal had to struggle for international recognition. He taught himself German and translated his own papers into French. Against this background, his achievement in establishing so many concepts fundamental to modern neuroscience is all the more extraordinary.

Jennifer Altman

Immunological ideas

N.A. Mitchison

The Immune System. By Rodney E. Langman. *Academic: 1989. Pp. 209. Hbk £31.50, \$49.95; pbk £14.50, \$22.95.*

IN 1968 Peter Bretscher and Melvin Cohn, one of the masters of modern immunology, formulated the associative-recognition or two-signal theory for self-non-self discrimination: a lymphocyte requires two signals in order to undergo productive activation, whereas a single signal results in the clonal deletion that underlies tolerance-of-self. Since then Cohn has collaborated with Rodney Langman, and this book, written by Langman but with a 33-page introductory essay by Cohn, gives a clear and punchy account of the ideas that have sprung from the theory.

Langman deals with the origin in evolution of tolerance, of B and T cells, of the major histocompatibility complex (MHC), and indeed of the whole immune system, and touches on interleukins, complement and other effector functions, and on the biochemistry of lymphocyte activation. The main themes, in addition to associative recognition, are the dual-recognition-single-receptor theory of recognition by T cells; the protection theory of establishing the antibody repertoire in two stages, by germ-line diversification followed by somatic mutation; and the adaptor theory of determining effector class (cytotoxic T-cells versus antibody) by suppressive regulation.

The book is not offered as a logical or comprehensive exposition of how the immune system works (for that I recommend my colleague Martin Raff's chapter in *Molecular Biology of the Cell* by Alberts *et al.*). Nor is it in any sense a review — the contributions of others are hardly acknowledged, there is no narrative to tell us what prompted Langman and Cohn to form their views, and no attempt is made to sort out the key observations. As such *The Immune System* will bemuse beginners and irritate the more experienced immunologist. On the other hand, for someone who already has a grasp of the subject, it is fun to watch the tide of immunological observations being accommodated within such a strong conceptual framework.

I find that framework weak at the following points. The account of evolution traces the immune system back to the defence system of invertebrates, rather than to their homotypic adhesion molecules, and the view of vertebrate evolution starts from terrestrialism but nevertheless admits the existence of sharks. The discussion of evolutionary waste omits the

highest cost of evolution, namely the maintenance of polymorphism. The contention that antigen processing and presentation by MHC molecules can be dispensed with in recognition by T cells is doubtful (the implications of the Bjorkman-Wiley structure do not seem to have reached La Jolla). There is a failure to realize how different modes of loading underlie the difference between MHC Class I and Class II molecules and to comprehend the importance of suppression for the control of hypersensitivity. The new ideas about staging in the thymus (the CD4/CD8 double-positive cell as the target of selection) are neglected. Finally, and most seriously, a key idea not mentioned in the book is that by relegating to T cells the job of maintaining self-tolerance, B cells become free to hypermutate.

On the positive side, Cohn and his colleagues were the first to realize the importance of the signals that lymphocytes receive through molecules other than their antigen-binding receptors. Just how important those signals are is evident in recent experiments demonstrating that cloned T-cells become unresponsive if stimulated in their absence. The fact that this book continues to present them in a much over-simplified fashion as a single second signal hardly detracts from the credit. Cohn also got the balance between germ-line and somatic diversification right at an early stage, and the expanded account presented here of the logic of diversification makes interesting reading. But I wonder whether the effort is justified: perhaps it makes more sense to sit back and wait until the astonishing range of diversification mechanisms adopted by different groups of vertebrates has been more fully explored.

I agree with the point of view expressed by Cohn in his introduction (and by Burnet before him), that ideas matter in immunology, perhaps more so than in other branches of biology, and that their most important function is in organizing otherwise incoherent sets of observations. But they need to be used with discretion and flexibility. Cohn criticizes others for their intellectual rigidity over the years, but it seems to me that the account of immunology that is presented here lays Cohn and Langman open to precisely that charge. □

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New in paperback

● *A Passion for Science*, by Lewis Wolpert and Alison Richards (Oxford University Press, £4.95). For review see *Nature* 334, 112 (1988).

● *Who Got Einstein's Office? Eccentricity and Genius at the Institute for Advanced Study*, by Ed Regis (Penguin, £5.99). For review see *Nature* 332, 497 (1988).

Shell show

Tim Halliday

Turtles of the World. By Carl H. Ernst and Roger W. Barbour. *Smithsonian Institution Press*: 1989. Pp. 313. \$45.

SUCH is the rate of decline of turtles and tortoises that we may never have the opportunity to learn more about many of them than is presented in *Turtles of the World*. The pictures shown below are reproduced from the book, which is the first to provide comprehensive coverage of the world's 257 turtle species, a group of reptiles with a 200-million-year history.

Each species is covered under the headings of description, distribution, habitat and natural history. There is considerable variation in the amount of material presented under the last heading, reflecting the fact that the behaviour and ecology of some species have been studied intensively, whereas little is known about others. The species entries are organized taxonomically and the book also includes detailed keys to identification at the levels of family, genus and species.

The most distinctive feature of turtles is the shell which, for most species, provides a protective haven into which the limbs and head can be withdrawn when the animal is threatened. There is considerable variation, however, in its form and protective role. In the softshell turtles, such as *Apalone spinifera* and *Cycloderma frenatum*, the amount of bone in the shell

is greatly reduced, and the main means of defence is to burrow in sand or mud. The head of the Asian big-headed turtle *Platysternon megacephalum* is so large that it cannot be withdrawn into the shell; this species appears to compensate for its greater vulnerability by being nocturnal. The evil-tempered Mexican giant musk turtle *Staurotypus triporcatus* is able to keep its jaws open even when the head is withdrawn, making it a formidable adversary. The shells of many turtles are beautifully patterned and coloured, as in the spotted turtle *Clemmys guttata*.

Turtles face a number of threats. Many species make very good eating, others have been adversely affected by insecticides and herbicides. They frequently suffer heavy casualties on roads and the populations of several species have been severely reduced by the pet trade. Being generally long-lived and slow-breeding, turtles and tortoises are also vulnerable to high levels of hunting. Some are at their most exposed when the young leave the eggs, dig their way out of the earth and find their way to water. Aquatic species have to wait until conditions are wet and the young of the Australian giant snake-necked turtle *Chelodina expansa* have been known to be trapped underground for nearly two years by drought.

Turtles of the World represents an impressive piece of scholarship. For the specialist it will prove to be an invaluable source of information for many years. □

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Platysternon megacephalum



Cycloderma frenatum



Staurotypus triporcatus



Clemmys guttata



Apalone spinifera



Chelodina expansa

The Almanac man

Jack Meadows

Nevil Maskelyne: The Seaman's Astronomer. By Derek Howse. Cambridge University Press: 1989. Pp. 280. £40, \$59.50.

THE Royal Observatory at Greenwich played a central role in British astronomy for three centuries. Yet there are few modern biographies of the Astronomers Royal, those king-pins of the astronomical establishment. This may be because the kind of work that traditionally occupied much of their time — positional astronomy — has had a rather poor press over the past few decades. One of the marvellous quotes in Derek Howse's book comes from an Assistant at the Observatory in the eighteenth century:

Nothing can exceed the tediousness and ennui of the life the assistant leads in this place, excluded from all society. . . . Here forlorn, he spends days, weeks, and months, in the same long wearisome computations . . . He is also up frequently three or four times in the night.

These bitter comments reflect only too well the image that a modern astrophysi-

cist has of the olden days. This new biography of the fifth Astronomer Royal, Nevil Maskelyne, shows that both he, and most of his assistants, had much more interesting lives. One reason was that astronomy in the eighteenth century had a greater strategic significance than it has today. As the book's subtitle indicates, astronomers were the chief advisors to governments on how to navigate accurately at sea — a limiting factor for all long voyages at that time. Although the Royal Observatory had been set up by Charles II primarily to find a means of determining longitude at sea, that aim had still not been achieved by the mid-eighteenth century. Maskelyne's historical importance was that two practical navigational techniques were developed during his period in office. His own contribution was the

development of an almanac which allowed position at sea to be determined from the position of the Moon in the sky. This *Nautical Almanac* was an essential item for ocean-going ships until after the Second World War.

As befitted his post, Maskelyne had some involvement in most of the principal astronomical advances in the latter half of the eighteenth century. His best-known work was his measurements of Schiehallion, a mountain in Perthshire, which he used as a means of estimating the density of the Earth. As Howse says, the analysis may have involved the earliest application of contour lines (although the French might dispute this). In another chapter, we are given the full story of Maskelyne's

uncomprehending discovery of the personal equation. Similar fascinating details occur throughout the book — for example, Maskelyne's dealings with Captain Cook, Sir Joseph Banks and assorted quarrelsome clockmakers, or how Mason and Dixon came together to measure their line in the United States.

This is as good a scientific biography as one could wish to have — fully documented, well illustrated, yet written throughout with a light touch. It provides an excellent insight into the world of British astronomy some two centuries ago. □

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The good bits

William H. Press

The Turing Omnibus: 61 Excursions in Computer Science. By A.K. Dewdney. Computer Science Press: 1989. Pp. 415. Distributed by W.H. Freeman, \$24.95, £22.95.

WE at universities like to have some idea — if only of the vaguest sort — of what our colleagues in other departments actually do. Most of us have a faint glimmering of what biochemistry, say, is about, or sociology, or Sanskrit or statistics. But what of that brash new department, computer science?

A seemingly benign computer scientist was on one occasion approached at an interdisciplinary, and well-lubricated, academic function. "Just as physics studies reality", he declared, "computer science studies *artificial* reality". Somehow this is not the kind of explanation one is looking for.

Now comes A.K. Dewdney, a computer scientist at the University of Western Ontario, with just the kind of answers that we need. *The Turing Omnibus* is full of wit — both kinds — from the double-barrelled pun of its title, all the way through its 61 short chapters, each of which amounts to a short essay, five or six pages long, on a particular topic in computer science. The "good bits" of computer science, Professor Dewdney cannot resist calling them.

This is a light-hearted but by no means light-weight volume — a book that 'does' Gödel's theorem in seven pages is no easy read. Although there are few mathematical proofs as such, there are a lot of summaries of the essential arguments of important proofs, algorithms and constructions. Thus we have wonderfully concise discussions of language hierarchies (regular versus context-free and so on), error-correcting codes, NP-completeness,

tomographic algorithms, hashing, linear programming, minimum spanning trees, the knapsack problem, lambda calculus, neural nets — plus (literally) hundreds of additional concepts, because several ideas are frequently covered in a single short chapter. The "good bits", indeed! There is a detailed index. If its prose style had been turgid instead of informal, its visual design scholastic instead of amiable, *The Turing Omnibus* could have called itself *Short Encyclopaedia of Computer Science* and it would have elicited generally warm reviews.

Against this praiseworthy background one must, however, mention a serious deficiency: technical accuracy, or 'fact-checking', of the book is not up to the standard of a credible short encyclopaedia, or a first-rate textbook. For example, the discussion of probabilistic tests for primality, while it gets the spirit of the idea right, states the algorithm incorrectly. Another example: the account of cubic splines seems to miss the point that they are smooth in their *second* derivative, and that they are global, not local.

These kinds of slips — there are some others — make *The Turing Omnibus* much less useful than it might otherwise have been; researchers in related fields, or students, would be ill-advised to rely on it as a one-volume reference work (as the author suggests in his preface). Such a caveat in no way diminishes its charm and usefulness in a limited niche, however. It is nearly the perfect book for non-computer scientists who want to learn something about the field, for undergraduates who want a detailed snapshot of what their prospective major field is about, or even for the bright secondary school student whose imagination will be fired up by a book that shows that there can be diverse, deep *ideas* inside computers — and no small amount of challenging intellectual fun. □

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Nevil Maskelyne (1732 – 1811).

cist has of the olden days. This new biography of the fifth Astronomer Royal, Nevil Maskelyne, shows that both he, and most of his assistants, had much more interesting lives. One reason was that astronomy in the eighteenth century had a greater strategic significance than it has today. As the book's subtitle indicates, astronomers were the chief advisors to governments on how to navigate accurately at sea — a limiting factor for all long voyages at that time. Although the Royal Observatory had been set up by Charles II primarily to find a means of determining longitude at sea, that aim had still not been achieved by the mid-eighteenth century. Maskelyne's historical importance was that two practical navigational techniques were developed during his period in office. His own contribution was the

Adaptation, speciation and hybrid zones

N. H. Barton & G. M. Hewitt

Many species are divided into a mosaic of genetically distinct populations, separated by narrow zones of hybridization. Studies of hybrid zones allow us to quantify the genetic differences responsible for speciation, to measure the diffusion of genes between diverging taxa, and to understand the spread of alternative adaptations.

HYBRID zones are narrow regions in which genetically distinct populations meet, mate and produce hybrids^{1,2}. They are found in all major groups of higher organisms, and divide species into a patchwork; adjacent regions can be distinguished by every kind of character from nuclear and cytoplasmic DNA, allozymes and chromosomes to colour pattern, morphology and behaviour. Because the hybridizing populations exchange genes, and yet remain distinct, they have long perplexed taxonomists. A biological species is defined as an "actually or potentially interbreeding group of populations"³; any taxa that can produce viable and fertile F₂ generations or backcrosses are regarded as being of the same species. But taxa that remain distinct despite gene exchange have in fact been classified as separate species even by the originators of the biological species concept³⁻⁵. Thus there is a clash between two views of species; one is based on the pattern of gene flow, and the other on the maintenance of a cluster of phenotypes that is stable to invasion by foreign genes.

Although it can confuse attempts at rigid classification, the conflict between gene flow and selection towards alternative stable equilibria gives us insight into both processes. Hybrid zones are especially well suited for testing the view that adaptation involves a 'shifting balance' between selection on individuals, and selection on whole populations⁶. This view, which was first advanced by the influential American biologist Sewall Wright in the 1930s, holds that selection between individuals leads populations to local optima or 'adaptive peaks', but that further progress requires that different regions reach different peaks, and that superior peaks then spread through a form of group selection.

Hybrid zones are natural laboratories that can tell us about the nature and effects of differences among incipient species, and about how selection at different levels affects the spread of alternative adaptations. We will first summarize a survey of over 170 hybrid zones, emphasizing the common features shared across diverse groups. We will then show how mathematical models can be used to measure the genetic basis of reproductive isolation, and to understand the spread of different adaptive peaks.

General patterns

Populations separated by hybrid zones may differ greatly, or hardly at all. Sometimes only a few genes seem to be involved; for example, the races of the mimetic butterfly *Heliconius erato*, which hybridize in South America, differ by between one and six major genes affecting wing pattern. No biochemical divergence or reproductive incompatibility has been found⁷. In other cases, the taxa may differ in almost every respect. For example, the fire-bellied toads *Bombina orientalis* and *B. variegata* interbreed freely in a narrow zone that runs for 1,000 km through eastern Europe. But they differ in mating call, warning coloration, life history, preferred habitat, enzymes and mitochondrial (mt) DNA; their biochemical differences indicate that they have been diverging for 3-4 million years⁸.

Some patterns do emerge from this diversity. Most hybrid zones are narrow relative to the species' range, and to the distance moved in a generation, σ : the majority of zones are less than 50σ wide¹. (The dispersal rate, σ , is defined as the

standard deviation of the distance between parent and offspring along a chosen axis; it has units of distance time^{-1/2}; $\sigma^2/2$ is analogous to a diffusion coefficient.) In the *viatica* group of morabine grasshoppers, for example, one chromosome arrangement may be replaced by another over less than 20 m (refs 9, 10). In the land snail *Cerion*, different shell morphs can be separated by as little as 100 m (ref. 11).

Most zones involve changes in a variety of characters, and so consist of a cluster of parallel gradients in gene frequencies (these gradients are termed 'clines'). It is not simply because complex zones are more easily detected that multiple differences are seen so often. On the contrary, zones that are first identified through characters such as plumage or chromosome arrangement are usually found later to involve biochemical differences. On average, 18% of enzyme loci show substantial frequency differences, and Nei's genetic distance averages 0.26 (samples of 46 and 34 zones, respectively). This suggests that divergence is ancient—~1 million years if a rough molecular clock is assumed¹². Because hybridization may dilute biochemical differences, this could be an underestimate.

The different clines which make up a hybrid zone usually coincide. For example, where the toads *Bombina orientalis* and *B. variegata* meet in southern Poland, clines in allozymes, belly pattern, mating call and mtDNA differ in position by less than 30% of their average width, and vary in width by at most 27% (ref. 8, and similar additional data; cline width is defined throughout as $1/\text{maximum gradient}$). Of all the continuous transects studied so far, characters encoded by nuclear DNA are significantly displaced in only four¹³. Hybrids can be found in mosaic distribution, such as those of the crickets *Gryllus firmus/pennsylvanicus* in Connecticut¹⁴. Even here, however, the correlation between different characters can be quite close. (The average pairwise correlation among five biochemical markers in 16 sites is $r = 0.49$.)

Mitochondrial DNA often shows a different pattern. For example, all the nuclear genes that distinguish the house mice *Mus musculus musculus* from *M. m. domesticus* change together in Jutland¹⁵. By contrast, one mtDNA from *M. m. domesticus* has penetrated about 750 km north into Sweden¹⁶ (although this has not happened in other parts of the zone¹⁷). Several other cases of such discordance are also known (for example see refs 18, 19). Discordance may arise by introgression through stable hybrid zones, or from occasional founder effects^{10,16}. Mitochondrial DNA may differ from nuclear genes because selective interactions between mitochondrial and nuclear genes are weaker than those among nuclear genes, and because mitochondria segregate independently of chromosomes¹⁹. The difference, however, may appear simply because phylogenies of mitochondrial sequences can be determined relatively easily. Moreover, the different patterns are not incompatible: recent differences may change together in a sharp hybrid zone, whereas more ancient differences may have introgressed^{17,18}.

There are often strong associations ('linkage disequilibria') between genes, chromosomes and morphological characters coming from the same parental populations. These are generated by the continual diffusion of parental combinations of genes into the centre of the zone and are broken down by recombina-

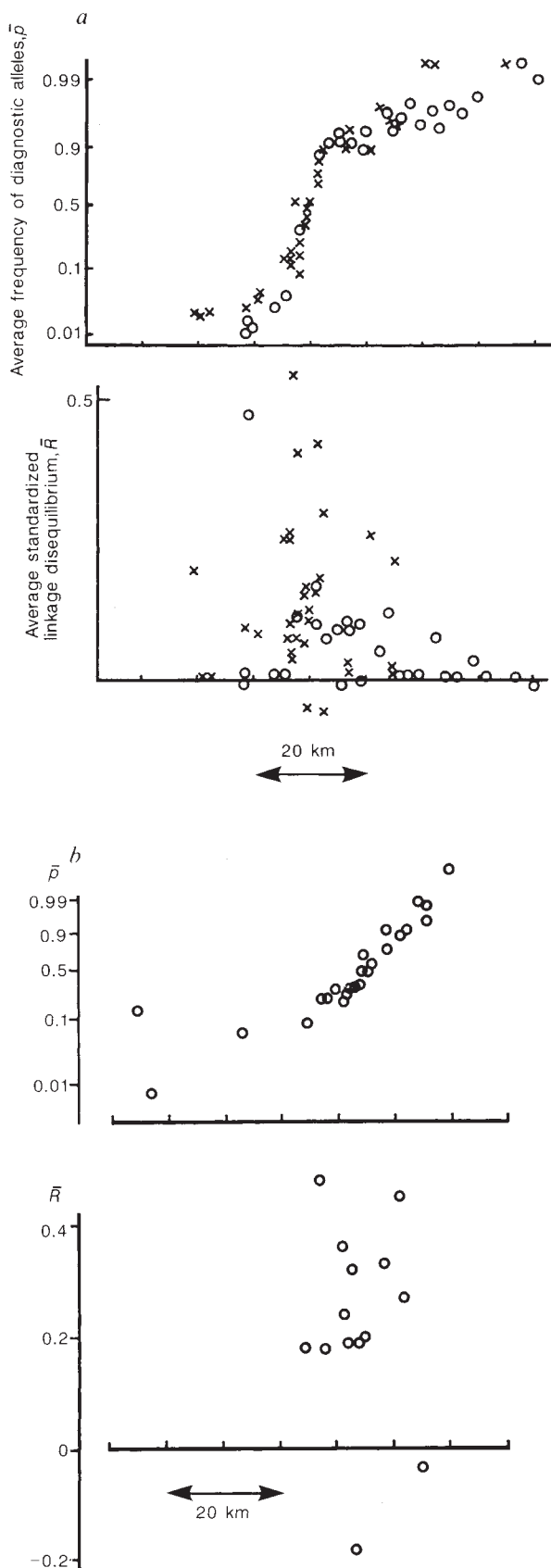


FIG. 1 Graphs of the average frequency of diagnostic alleles, $\bar{p}$, and the average standardized linkage disequilibrium ($\bar{R} = D/\sqrt{p_1q_1p_2q_2}$) across two hybrid zones. *a* Fire-bellied toads, *Bombina bombina/variiegata* in southern Poland (data from ref. 8, together with new data; six enzyme loci. Crosses: Cracow transect. Circles: Przemyśl transect). *b*, Butterflies *Heliconius erato* in Peru; three wing-pattern loci (Mallet, personal communication).

tion and segregation in hybrids. Disequilibria can hence give an estimate of the rate of diffusion, σ (ref. 20). For example, where the Tarapoto and Amazonian races of the butterfly *Heliconius erato* meet and hybridize in Peru, there are significant disequilibria between all three loci involved, even though these are on different chromosomes (Fig. 1*b*). These give an estimated dispersal rate of $\sigma \approx 2.4$ km generation^{-1/2}. This indirect method of measuring genic diffusion gives values higher than those from studies of individual movement ($\sigma \approx 0.3$ km generation^{-1/2} in *H. erato*²¹). Because direct measurements often miss long-distance migrants and sporadic population movements²², the indirect measures may be more accurate. The discrepancy could also arise if associations are built up by selection for particular gene combinations, as well as by diffusion. But the error should not be more than twofold even for a pair of clines maintained entirely by selection for particular gene combinations; with more loci the effect becomes negligible²³.

What maintains hybrid zones?

We believe that most hybrid zones are in fact 'tension zones'²⁴, which we define as clines maintained by a balance between random dispersal and selection against hybrids¹. Before describing the evolutionary significance of tension zones, we will briefly explain our reasons for this opinion.

Most zones involve strong selection, as is made manifest by the finding of hybrid inviability or sterility in the field²⁵⁻²⁹ or laboratory^{8,30-34}, the elimination of individuals³⁵ or chromosomes³¹ moved across the zone, comparison between age classes³³, or, less directly, chromosome aberrations^{31,36,37} or morphological anomalies⁸ in hybrids. Evidence also comes from the strong associations between different loci, which must ultimately be maintained by selection—mixing can only produce strong and persistent linkage disequilibria if selection maintains steep gradients in gene frequencies. Finally, clines are often narrow relative to the dispersal range. For example, in the hybrid zone between the grasshoppers *Chorthippus parallelus parallelus* and *C. p. erythropus*, the nucleolar organizer changes over less than 600 m at Col de la Quillane, and parts of the song of the grasshoppers also change sharply³⁸. These races have been in contact for at least 8,000 generations, a period long enough to produce a cline more than 7 km wide if they had been mixing at the observed rate of $\sigma \approx 30$ m in a generation. Selection must therefore be acting on these characters, a conclusion supported by the fact that other clines in the same hybrid zone are much wider (~ 15 km for allozymes, and ~ 4 km for the stridulatory pegs¹³).

Is selection in hybrid zones a direct response to environmental variation, or does it instead reflect the intrinsic inferiority of hybrids? It is conceivable that selection actually favours hybrid genotypes in particular environments³⁹. But this would not account for the typical composition of hybrid populations: one would expect that only a few hybrid genotypes would dominate, as when hybrids reproduce parthenogenetically or by self-fertilization (for examples see refs 40, 41). By contrast, hybrid zones contain a mix of gene combinations, with associations of similar magnitude between all pairs of genes (for example, *Heliconius* (Fig. 1*b*); see also refs 15, 20, 42, 43). The superiority of hybrids in particular environments also cannot account for the long, narrow strips of roughly even width that are typical of most zones (see for example Fig. 3*a*).

It is difficult to distinguish between other possibilities. The selection that maintains a hybrid zone could favour different alleles in different places, or it could act against hybrids. For either case, the shape of the cline would be similar (Fig. 2): alleles that cross the cline would be eliminated either because they were in the wrong environment, or because they were combined with the wrong genes. Frequency-dependent selection against rare alleles would have effects similar to those of selection against hybrids^{21,44,45}. Where zones in several species coincide, interspecific interactions might also maintain clines.

Finally, all these kinds of selection might act in the same zone. For example, selection against heterozygotes, recombinants and rare morphs, together with interspecific mimicry, act to maintain homogeneous warning patterns in *Heliconius* butterflies²¹. (See also refs 8, 43, 46.)

Although there is evidence for adaptation to local environment from many zones^{1,2,13}, selection against hybrids is probably more important in maintaining them. First, different clines often coincide very closely; if each allele were to respond directly to the environment, such geographic concordance would not be expected, even allowing for the effects of linkage disequilibrium⁴⁷. Second, even if differences were established as an adaptive response to environmental differences, subsequent coadaptation would be likely^{48,49}. For example, the evolution of tolerance to heavy metals in plants is sometimes accompanied by partial reproductive isolation⁵⁰. In the monkey flower *Mimulus guttatus*, a perennial plant found, in addition to other places, along the banks of streams on disused open-cast mines in North America, one tolerance allele is incompatible with an allele at another locus in the original population⁵¹. Although it is difficult to understand just how incompatibility between differently adapted races arose⁵², it seems likely that clines that are initially maintained solely by differential adaptation will, as more differences accumulate, develop into tension zones which can be maintained even without the original environmental difference^{1,2,13}.

The mathematics of tension zones

All but the simplest types of natural selection lead to multiple stable equilibria: populations may respond to the same selection pressures by evolving different combinations of genes. At the boundary between populations in different stable states, a bal-

ance between random dispersal and the disruptive attractions of alternative equilibria usually maintains a narrow cline.

Bazykin⁵³ suggested the simplest model of a tension zone. Heterozygotes have fitness $1 - s$ relative to either homozygote. If the allele frequency is p , the change in allele frequency is then (for small s)

$$\frac{\partial p}{\partial t} = \frac{\sigma^2}{2} \frac{\partial^2 p}{\partial x^2} + spq(p - q) \quad (1)$$

where $p + q = 1$. This equation has the solution $p = 1/(1 + \exp[-(x - x_0)/l])$, where $l = \sqrt{\sigma^2/2s}$ is the characteristic distance over which selection changes allele frequencies; the cline width is $4l$.

In this model the environment is homogeneous and the homozygotes have equal fitnesses, so the cline can form anywhere (x_0), and the equation has a family of neutrally stable solutions. These are known as 'topological solitons'⁵⁴—solitary waves set up at the boundary between two regions constrained to be in different states. The same phenomenon (often the same equation) arises in many other fields (see refs 55–59.) Bazykin's model is based on a special kind of reaction–diffusion equation⁶⁰, in which attention is centred not on the spontaneous formation of pattern from a homogeneous field (as in Turing's model⁶¹), but on the interaction between areas which have moved to different states. Indeed, the boundary between regions at high and low concentrations in the Turing model behaves in a similar way to the tension zone.

Barriers to gene flow

Although gene frequency can change abruptly across the centre of a hybrid zone, foreign alleles can still penetrate far into either side. This gene flow between distinct populations binds them together into one biological species. In *Bombina*, although the average frequency of diagnostic enzyme markers changes by ~90% over 6 km, foreign alleles have been found up to 38 km from the centre of the zone (Fig. 1a). If this was due simply to a few long-distance migrants, parental and F1 genotypes would be expected to be found well outside the zone; strong linkage disequilibrium would also be expected there. This is not the case for *Bombina*. In other studies data are not yet available to rule out this possibility.

Smooth sigmoid clines are expected for introgressing alleles that are diffusing freely or are subject to only weak selection (Fig. 2). A barrier to gene flow, caused for example by a physical obstacle, will produce a step in gene frequency, Δp , at the centre of the cline proportional to the gradient ($\partial p/\partial x$) on either side⁶². The strength of the barrier can be estimated from the shape of the cline as $B = \Delta p/(\partial p/\partial x)$. This measure has the dimensions of distance, and its magnitude relative to the dispersal rate (B/σ) determines the delay to the spread of genes across the barrier. Although neutral alleles can suffer a lengthy delay [$T \approx (B/\sigma)^2$], alleles with even a slight advantage (S) will hardly be impeded ($T \approx \log[(B/\sigma)^2 \pi S/2]/2S$). This is because once a few of them penetrate the barrier, they will increase exponentially and spread to fixation⁶³. In making these comparisons it is important to note that even without barriers, neutral alleles will take ~10,000 generations to spread over ~100 σ by diffusion alone. Because species' ranges are unlikely to remain stable for such long periods, neutral variants are much more likely to be carried long distances by gross population movements ('convection') than by local diffusion^{1,3,22}.

For example, the strength of the barrier to gene flow (B) from *Bombina bombina* to *B. variegata* is equivalent to 51 km of unimpeded habitat (support limits, 22–81 km). This compares with a dispersal rate (σ) of 0.99 km, and a cline width (w) of 6.1 km. (The figures here differ from those in ref. 8 because new data have since been included.) The barrier would delay neutral alleles for ~2,700 generations, which is about the same as the time for which the present hybrid zone has existed. The barrier

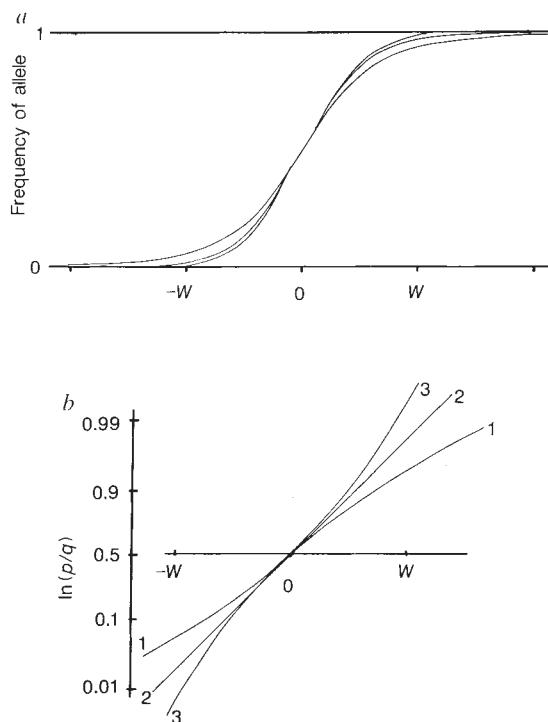


FIG. 2 a, Clines maintained by a balance between dispersal and selection all have similar sigmoidal shapes. b, Transformation to a logit scale [$\ln(p/q)$] reveals differences in the shape of the tails; because these involve extreme gene frequencies, however, they can only be detected with large samples. Cline width is defined as $w = 1/(\text{maximum gradient})$; the graphs are scaled so that all clines have the same width. Three cases are shown: (1) selection, $+s$ on one side of a sharp environmental change, $-s$ on the other ($w = \sqrt{6}/l$), (2) heterozygote disadvantage ($w = 4/l$); and (3) introgression of neutral alleles for T generations ($w = \sqrt{4\pi T}$, where $l = \sigma/\sqrt{2}$).

would, however, delay an allele with an advantage of 1% for only ~200 generations.

The barriers to gene flow revealed by sharp steps at the centre of many hybrid zones can be explained by linkage disequilibrium. At the centre, each allele is associated with others from its ancestral population. Selection acts, therefore, not on individual loci, but on correlated sets of loci. If the net selection pressure is great enough relative to the rates of recombination and segregation, strong selection can be induced on each locus by a 'hitch-hiking' effect⁶³. A sharp gradient is produced, which in turn generates stronger linkage disequilibria and induces yet stronger selection²³. But as foreign alleles introgress through the zone they will recombine into the new genetic background, and

so will only be subject to selection arising directly from their own effects²³. This argument leads to a general expression for the barrier strength, B , in terms of measurable quantities: mean fitness, average recombination rate and cline shape. When applied to *Bombina*⁸, this expression shows that the barrier to gene flow can be explained by a reduction in mean fitness at the centre of the hybrid zone to 58% (support limits, 54–68%). Because this reduction is much larger than the selection required to maintain associations between particular pairs of enzyme loci, selection must act on many genes. The number of genes responsible for reproductive isolation between *B. bombina* and *B. variegata* can be estimated from this argument to be ~55 (support limits, 26–88). This estimate provides a rare insight into the number of loci involved in speciation.

The movement of tension zones

To understand how species form, and to judge the importance of Wright's shifting-balance theory in adaptation, it is crucial to know whether a tension zone can move from where it was first formed and, if so, what factors are important in determining its position. Because tension zones are maintained by interactions between genes, rather than in direct response to a heterogeneous environment, they are not held in any fixed position. They can be perturbed by local variations in population structure (that is, by population density and dispersal rate), and by adaptation to local environments.

Different kinds of perturbation can be summarized in a function ($\bar{H}$) of zone position, which tends to a minimum. In the panel on the left, we show how this potential function can be derived for the simple case of selection, s , against heterozygotes that we described in equation 1. Terms are added to allow for a selective advantage $2S$ of one homozygote over the other, and for variations in 'neighbourhood size' ($N = 4\pi\rho\sigma^2$, the product of density ρ and the dispersal rate). This function is easy to generalize to other selection schemes. The tension zone will move to minimize its length, and hence to eliminate any bulges in the zone. It will also move towards regions of low neighbourhood size, and so will come to rest at physical barriers to gene flow. Finally, any allele with a superior homozygote will tend to spread. These effects can be illustrated by a modified version of Wright's 'adaptive landscape', in which neighbourhood size (squared) is plotted against position; the tension zone can be pictured as a heavy elastic ring, which tends to roll down the surface, but also to contract into a smooth curve. If the equilibrium contained within the ring is superior, there will be an additional outward pressure on the ring.

In spite of the complexity of the forces that can move tension zones, some generalizations can be made. Because density and dispersal may vary greatly, even over short distances, the potential surface will be rough. Thus, unless one equilibrium is much superior to another ($S \approx s$), the zone will be trapped by local barriers. The zone could only move far from where it first formed through convective movements of the whole population. In the extreme, the species might become extinct over large areas; when conditions improved, the zone would form again in a new position. In less drastic cases, fluctuations in population density might push zones from place to place.

The two chromosomal races of the grasshopper *Podisma pedestris* may be considered as an example. These meet along the main ridge of the Alpes Maritimes (Fig. 3a), close to the line along which southern and northern populations are likely to have met after the last glaciation^{13,28,64}. The zone fluctuates by a few kilometres on either side of the ridge, so that the races do not necessarily meet exactly at high passes (Fig. 3b–d). On a smaller scale, the zone usually runs along cliffs, streams, grazed areas, or other inhospitable regions. In at least two places, however, it bulges outwards (Fig. 3b, lower left; d, upper right). These anomalies can be explained by the local distribution of grasshoppers—the pressure of numbers on the concave side of the bulge balances the contraction of the tension zone⁶⁴.

MOVEMENT OF TENSION ZONES

THE effects of all the forces that perturb a tension zone can be summarized in a single potential, $\bar{H}$, which tends to a minimum. It is calculated relative to a natural geometry, with scales set relative to local rates of dispersal and selection. Suppose that genes move across a rough two-dimensional habitat. Dispersal may be anisotropic, in which case the dispersal rate σ^2 becomes a 2×2 matrix, with elements σ^{ij} giving the covariance between the distance moved in directions i and j . The dispersal matrix may vary between times and between places; this can be represented by measuring distance relative to the ratio between dispersal and selection against heterozygotes, and time relative to $1/s$. This gives a new coordinate system, in which the distance between two nearby points dx^i apart is $dX^2 = dx^i g_{ij} dx^j$ and the time interval is $dT = s dt$. A new (non-euclidean) geometry is defined by the metric $g^{ij} = 2\sigma^{ij}/s$ (tensor notation is used here: g_{ij} is the inverse of g^{ij}). Terms must also be added that represent the effects of variations in neighbourhood size ($N = 4\pi\rho\sigma^2$), and an advantage of one homozygote over the other⁷². Equation 1 becomes.

$$\dot{p} = \nabla^2 p + \nabla \log(N^2) \cdot \nabla p + pq(p - q + S/s) \quad (1.1)$$

where ∇^2 is the Laplacian, which replaces $\partial^2 p / \partial x^2$ in two dimensions, and $\dot{p}$ is the differential with respect to (rescaled) time. Equation (1.1) can be rewritten in terms of a potential H (a rescaled version of the potential in ref. 72):

$$\dot{p} = -\frac{1}{N^2} \frac{\partial H}{\partial p} \quad (1.2)$$

where

$$H = \int \frac{N^2}{2} (\nabla p \cdot \nabla p + p^2 q^2 + (S/6s)(p - q)(1 + 2pq)) dX$$

If inhomogeneities are small, then we can assume that in most places the population is close to one or other equilibrium. The boundary between regions containing different alleles is then still approximated by the solution to equation (1), and will move slowly. Equation (1.2) can now be simplified, because only the position of the boundary need be followed, rather than the whole field of gene frequencies. The full potential H is replaced by $\bar{H}$, which is calculated on the assumption that the tension zone is close to its unperturbed sigmoid shape. If we measure distance along the zone by z , and if $y(z, t)$ is the distance between the centres of the zone at z at times 0 and t , then the rate of movement is (by the same method as in ref. 72)

$$\dot{y} = -\frac{6}{N^2} \frac{\partial \bar{H}}{\partial y} \quad (1.3)$$

where

$$\bar{H} = \int \frac{N^2}{6} dz - \int \frac{N^2 S}{6s} dA.$$

This equation is a spatial analogue of Wright's formula for the evolution of a single panmictic population

$$\partial p / \partial t = (pq/2)(\partial \log(\bar{W}) / \partial p).$$

The first term is an integral around the zone, and is proportional to the length of the zone; the second term is an integral over the area occupied by the allele with advantage S . In a finite population, this equation must be modified to take account of the fluctuations caused by random drift, which will tend to make the zone expand⁶⁵.

Speciation and the shifting balance

The movement of tension zones helps to determine the pattern of speciation, and the way species evolve genotypes that are better adapted. Because they reveal the extent to which species are divided into regions near different equilibria, tension zones are closely related to Wright's model of a shifting balance between intrapopulation selection, random drift and interpopulation selection, which involves transitions between different stable equilibria.

A central tenet of evolutionary biology has been that gene flow greatly impedes divergence, so that species can only form in geographic isolation³. But the existence of sharp, stable clines and hybrid zones in the face of free dispersal shows that gene flow need not destroy spatial divergence. The crucial question is whether reproductive isolation can originate within a continuous population. If a new equilibrium is to be established by random drift, a population must move to this state in some small region. The new state will be separated from the old by a tension

zone, and it must, if speciation is to proceed, expand outwards until it covers an area large enough to resist swamping from the outside (Fig. 4a). The probability of a shift (per unit time and per unit area) can be calculated using methods adapted from quantum field theory^{59,65}. If neighbourhood size is small, and the new equilibrium is more attractive than the old, this probability can be appreciable (Fig. 4b). Even if the chance of any one mutation being fixed is small, the net probability summed over a large species range can easily account for observed rates of speciation and chromosomal evolution⁶⁶.

Similar arguments apply to shifts in populations subdivided into discrete demes⁶⁶. The dynamics of tension zones is such that sampling drift can cause divergence despite free gene flow. Reproductive isolation, however, can also be caused by geographical or temporal changes in selection in space and time (for example, *Partula*⁴⁵), or by the accumulation of incompatible mutations (for example, *Sorex*³⁶; see also ref. 52). These mechanisms may be more important than random drift; unfortunately, it is much harder to distinguish between possible mechanisms of speciation than to find the present nature and effects of species differences.

The abundance of hybrid zones tells us that populations can, and have, shifted between alternative stable combinations of

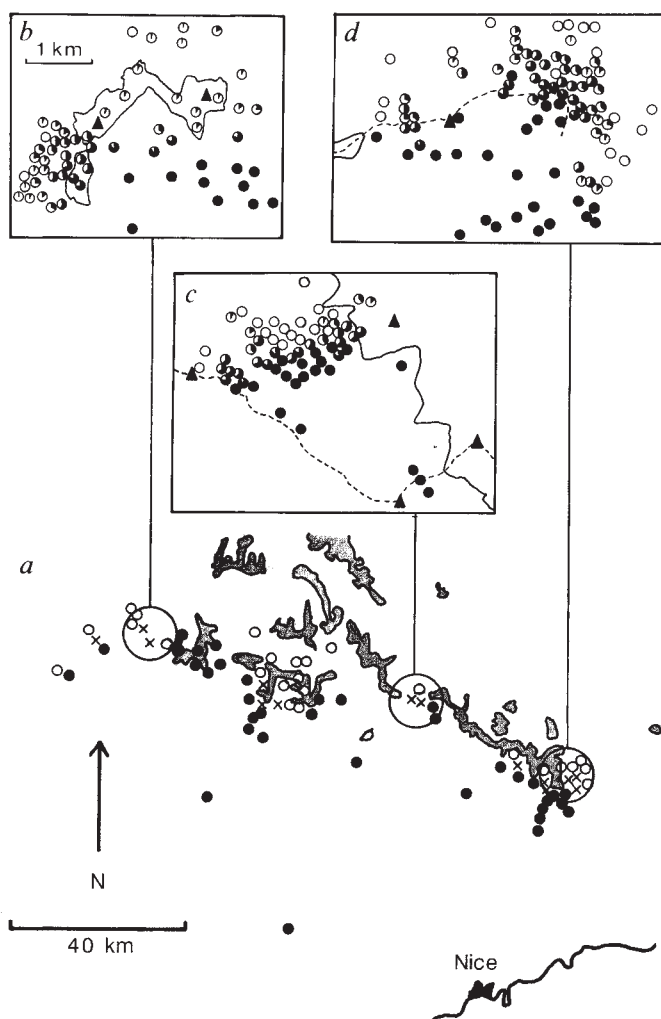


FIG. 3 a, The tension zone that separates two chromosomal races in the grasshopper *Podisma pedestris* runs near to the main ridge of the Alpes Maritimes. Open circles, samples fixed for the ancestral karyotype; filled circles, samples fixed for a Robertsonian fusion between the X chromosome and an autosome; crosses, mixed samples. Shaded areas are higher than 2,500 m b-d. Detailed transects show that the zone fluctuates a few kilometres on either side of the main ridge. The solid line shows the 2,500-m contour (except in b, where it shows the 1,900-m contour). All insets are to the same scale. The dotted line shows the main ridge, and triangles represent peaks. The pie diagrams show the frequency of the fusion in samples of ~20 males. b, Seyne; c, Col de la Lombarde; d, Vallon de Fontanalbe.

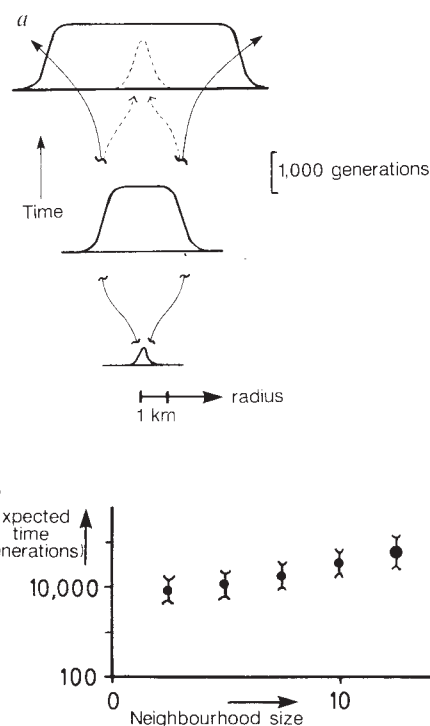


FIG. 4 a, The establishment of a new adaptive peak in a two-dimensional habitat. Disruptive selection acts on a polygenic character; the new peak gives a mean fitness that is 1.33% greater than that of the old peak, and the two peaks are separated by a fitness trough of depth 1.98%. A chromosome rearrangement with fitnesses 1:0.82:1.04 would follow a similar path. The dispersal rate is $\sigma=100$ m in a generation. The most probable path for a transition consists of an initial movement out to the unstable equilibrium, taking ~1,000 generations. The population may then fall back to the original equilibrium (dashed line), or the bubble may expand out to cover the entire range, eventually reaching a constant velocity of 3.2 m per generation. The two pairs of curves headed by arrows plot the radius of the bubble against time, (from ref. 65). b, A chromosomal rearrangement has an appreciable chance of fixation in a two dimensional habitat, provided that the new homozygote confers a substantial advantage, and that the neighbourhood size is not too large. The graph shows expected time (± 2 s.e.m.) before the new karyotype is fixed, plotted against neighbourhood size. Results are from a simulation of 10×10 demes, with $\sigma=0.45$ deme spacings. Fitnesses are 1:0.94:1.12, and the mutation rate is $\mu=10^{-5}$.

genes. Wright⁶ realized that mass selection would bring a large random-mating population to a nearby equilibrium, but not to the global optimum. This is the central obstacle to any optimization algorithm⁶⁷. Wright argued that evolutionary advance would be most rapid in a population spread over a large area, so that different gene combinations could be tried out in different places. Fitter combinations of genes would be more likely to be established, and would spread at the expense of others. Because selection is between different equilibria, selection could favour traits that are advantageous to the whole population, rather than to individuals. This is a form of 'group selection' that includes a powerful mechanism for generating and maintaining spatial variation, and that does not act in opposition to natural selection. To this extent, Wright's view is more plausible than traditional models for the evolution of altruistic traits⁶⁸⁻⁷⁰. Explicit models show that fitter combinations of genes are indeed more likely to be established⁷¹, particularly in a continuous population^{65,71}. Although an inferior equilibrium might be reached by chance in a single isolate⁷¹, this can never happen in a broad continuum: the inferior state will be swamped.

It is possible to estimate the relative importance of individual and group selection. To illustrate this, suppose that an increase in homozygote fitness by a factor of $1 + 2S$ causes the population density to increase by the same factor. Then, the tension zone will advance both because of the asymmetric selection between individuals, and because of the greater flow of genes out of areas containing the superior homozygote and hence having greater density. It is this latter effect which we see as a form of group selection. The rate of advance due to individual selection is $S/2s$ in scaled units, whereas the contribution of group selection is $S/5$, which must be smaller. Similar examples lead to the generalization that individual selection is usually stronger than group selection⁷². A similar conclusion has been reached for a population subdivided into demes, and subject to extinction and recolonization⁷¹.

There are other major obstacles to adaptation through the shifting balance. Equilibria can gain an advantage for arbitrary and non-adaptive reasons: dominant alleles²¹ and alleles giving increased dispersal⁷² tend to spread. More importantly, gene combinations that happen to coincide with regions of population expansion will gain an undue advantage. For example, the positions of most European hybrid zones are thought to be determined by postglacial expansion from small refugia^{2,13}. The gene combinations that happened to be in those refugia will be greatly over-represented. This process is analogous to sampling drift, but acts at a higher level. It will overwhelm any systematic group selection favouring new equilibria, simply because of the small number of independently expanding and contracting regions. A further problem is that tension zones involving one set of characters are usually clumped with zones involving others. Once independent zones are brought together, by co-adaptation or by a common response to population movements, they can only be separated by drastic events. Although tension zones can in principle pass through each other, producing a recombinant population, we know of only one clear example where this has taken place (*Warramaba viatica*; ref. 10). Thus, for the same reasons that selection on small asexual populations, or between whole species, is ineffective, Wright's shifting balance is unlikely to be a major cause of adaptation. Nevertheless, the processes that Wright analysed are essential to divergence and speciation.

Conclusion

It is extremely difficult to estimate selection pressures, rates of gene flow, and gene numbers from laboratory crosses or field manipulations. Studies of hybrid zones that include several markers and enough samples from a continuous transect are a uniquely valuable source of such information^{8,20,21,32,33,42}. The few cases that have been surveyed in sufficient detail indicate that reproductive isolation is caused by selection on large num-

bers of genes, and can pose a substantial barrier to gene exchange. More work is needed to show whether this remains true across a wider range of groups, and to find the precise mechanisms of isolation.

What is the genetic basis of the morphological and behavioural differences between hybridizing taxa? Is the spread of alternative adaptive peaks determined by their selective advantages, or by arbitrary changes in population structure? Does the increased variance in fitness in hybrid zones lead to changes in mating preferences or recombination rates? What is the effect of reduced fitness on the population size? Such questions touch on many controversies in evolutionary biology—hybrid zones provide a ready opportunity for their empirical investigation □

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ARTICLES

Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication

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Simian virus 40 large tumour antigen (T) is a replication origin binding protein required for viral DNA synthesis. Unphosphorylated T antigen is deficient in promoting DNA replication *in vitro* but can be activated by phosphorylation at residue threonine 124 by the cdc2 protein kinase. This observation demonstrates that T is regulated by phosphorylation and provides a model for cdc2 function in the control of DNA replication.

THE large tumour antigen (T) of simian virus 40 (SV40) is a multifunctional protein that plays diverse roles in the life cycle of the virus¹. The T antigen is required for initiation of viral DNA replication during lytic infections of permissive cells^{2,3} and also regulates both early and late viral gene transcription^{4–7}. T is known to regulate the two initial viral functions by direct interaction with two closely positioned sites (sites 1 and 2) within the SV40 genome^{8,9} (see Fig. 3). Binding to site 1 primarily acts to inhibit transcription of T (ref. 10), whereas site 2 is required for viral DNA replication^{11–15}. In addition to the DNA-binding properties of T, a variety of other biochemical activities have been associated with the protein, including DNA helicase¹⁶ and origin-specific unwinding activities¹⁷. T antigen also acts to induce and maintain a transformed phenotype in non-permissive cells¹, possibly by binding to cellular proteins such as p53 (ref. 18) and rb (ref. 19) that may normally act as negative regulators of growth. Only the role of T in viral DNA replication is considered here.

Cell-free lysates have been developed in which T antigen binds to the viral origin and promotes DNA replication^{14,20}. In such lysates the replication of SV40, or more generally plasmids carrying the viral replication origin, is absolutely dependent on exogenously added T but otherwise relies entirely on cellular factors. The ability of T to initiate DNA synthesis is influenced by its state of post-translational phosphorylation^{21,22}. T is phosphorylated in two regions, located in clusters at the N and C termini of the protein²³. Both of these lie outside a minimal region of the protein required for DNA binding²⁴ and may act both to activate or inhibit T-dependent replication^{21–28}.

We have taken advantage of T purified from *Escherichia coli* that is in an underphosphorylated state and is deficient in directing DNA replication²⁹, and find that the cdc2 protein

kinase, a critical regulator of the cell cycle in eukaryotes, phosphorylates T at threonine residue 124 (Thr 124) with great efficiency *in vitro*. This modification, which occurs *in vivo*²³ alters the DNA-binding pattern of the protein and activates T-dependent SV40 DNA replication. Our observations have implications for the role of cdc2 in the lytic cycle of the virus and possibly for the function of this protein kinase in cellular DNA replication.

Thr 124 phosphorylation

The cdc2 protein kinase is required for the initiation of DNA replication in two species of yeast, *Saccharomyces cerevisiae*³⁰ and *Schizosaccharomyces pombe*³¹, but its S-phase substrates remain unknown. During a search for possible substrates of cdc2 purified from HeLa cells³² we investigated the possibility that SV40 T antigen, which plays a critical role in viral replication, might be regulated by cdc2. As substrate, we initially used an N-terminal fragment of T (T 259) that retains origin-specific DNA-binding properties³³. T 259 was expressed in *E. coli* and purified by immunoaffinity chromatography (Fig. 1a, left panel). The purified protein was found to be readily phosphorylated by cdc2 (Fig. 1a, right panel). We estimate that ~20% of T molecules were modified during a prolonged incubation with the enzyme. Phosphoamino-acid analysis of the phosphorylated T revealed that only threonine residues were modified (Fig. 1b).

To map the threonine residue within T 259 phosphorylated by cdc2, the protein was cleaved with proteases (see Fig. 1, legend). The resulting peptides were resolved by gel electrophoresis³⁴. A major phosphate labelled band of relative molecular mass (M_r) ~3,500 (3.5K) was detected (Fig. 1c). The peptide was eluted from the gel and sequence analysis (see Fig. 1, legend) revealed the following: Asn-Leu-Phe-Cys-Ser-Glu-Glu-Met-Pro-Ser-Ser-Asp-Asp-Glu-Ala-Thr-Ala. Yields of further amino acids were below detectable limits. The first amino acid of this sequence corresponds to Asn 102 (ref. 1). Taking into account the apparent molecular mass of the proteolytic cleavage product and the specificity of trypsin and V8 proteases, we infer that the phosphopeptide spans residues Asn 102–Lys 131. This region contains two threonine residues, Thr 117 and Thr 124, of which Thr 124 is known to be phosphorylated *in vivo*²³. The region surrounding Thr 124 (His-Ser-Thr*-Pro-Pro-Lys-Lys-Lys-Arg-Lys) bears some similarity to sites within histone H1 that are phosphorylated by the growth-associated, or M-phase specific histone H1 kinase³⁵. This enzyme is thought to be the same as that used here^{32,36}.

To further examine the possibility that Thr 124 is the site of phosphorylation by cdc2, we prepared a mutant at this site by

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oligonucleotide mutagenesis^{37,38} and expressed the altered protein (T 259-Ala 124) in *E. coli*. The mutant protein was purified and found to be phosphorylated at least 200-fold less efficiently by cdc2 than is the wild-type protein (data not shown, but see Fig. 2). Taken together, these data demonstrate that Thr 124 is the site at which cdc2 phosphorylates T 259.

To stringently test whether the phosphorylation of T 259 by the HeLa cell preparation containing cdc2 was in fact due to this enzyme, T 259 kinase activity was assessed in every fraction of the final column (Mono-S) used to purify the cdc2. The profile of T 259 phosphorylation was compared with phosphorylation of histone H1, a known substrate of the enzyme^{32,36}. The elution profiles of T 259 and histone-H1 kinases overlapped closely (Fig. 2). Even with addition of equimolar amounts of protein, histone was more heavily phosphorylated than T 259 (it should be noted that histone contains multiple sites of cdc2 phosphorylation³⁵). Also, histone effectively competed for phosphorylation of the endogenous cyclin subunit of the kinase, p62 (see ref. 32), whereas T 259 did not (Fig. 2a). No column fraction phosphorylated the mutant T 259-Ala124 protein to an appreciable extent (Fig. 2).

Origin binding

The effect of phosphorylation by cdc2 on the DNA-binding properties of T 259 was examined using a modified McKay assay^{33,39}. In this assay an equimolar mixture of end-labelled DNA fragments containing either site 1, site 2, or the wild-type origin containing both sites 1 and 2 was incubated with T antigen. A monoclonal antibody (Pab419)⁴⁰ was used to precipitate T and any DNA fragment to which it may be bound. The labelled DNA was visualized by autoradiography (see legend to Fig. 3).

Unphosphorylated T 259 purified from *E. coli* has the highest

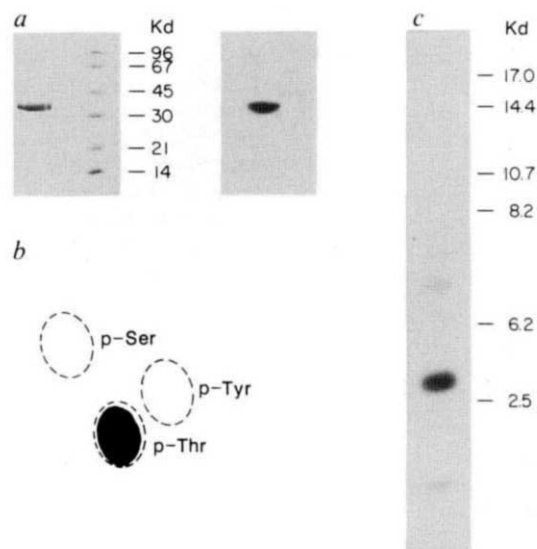
affinity for the wild-type fragment and binds preferentially to site 1 rather than site 2 (see refs 24, 33 and Fig. 3). Phosphorylation of T 259 by cdc2 strongly stimulated the ability of the protein to bind to the wild-type DNA fragment and reversed the order of preference of the unphosphorylated protein for binding. Quantitation of this effect by scintillation counting of excised gel bands revealed that phosphorylation of T 259 caused a twenty-fold increase in binding to the wild-type DNA and site 2-containing DNA fragment. Little, if any, effect on site 1 binding was observed.

To test whether these effects were a consequence of phosphorylation at Thr 124, the mutant protein (T 259-Ala 124) was exposed to cdc2 under standard phosphorylation conditions. The mutant protein binds to site 1 and the wild-type origin bearing DNA fragments with efficiency comparable to the native T 259, but its DNA-binding activity was unaffected by exposure to cdc2 (Fig. 3). This confirms that the altered properties of the wild-type T 259 following exposure to cdc2 is due to phosphorylation at Thr 124. The DNA-binding pattern of bacterial-derived T 259 that has been phosphorylated by cdc2 was indistinguishable from that of the same protein purified from mammalian cells^{24,33}, displaying a marked preference for binding to site 2 (within the core origin) compared to site 1 (within the transcriptional autoregulatory region).

In a further test, we investigated the effect of phosphorylating full-length T (T 708) with cdc2. Like T 259, T 708 purified from *E. coli* binds efficiently to site 1, but poorly to site 2 (ref. 29 and Fig. 4). After exposure of bacterial-derived T 708 to cdc2, site-2 binding was stimulated at least 10-fold (Fig. 4). Again this property mimics that of T 708 purified from mammalian cells rather than *E. coli* (Fig. 4). Exposure of mammalian T 708 to cdc2 resulted in no further stimulation of its site-2 DNA-binding

FIG. 1 Phosphorylation and phosphoamino-acid analysis of T 259. *a*, Left panel, Coomassie blue staining of immunoaffinity-purified T 259. Right panel, autoradiogram of T 259 phosphorylated by cdc2. *b*, 2-D-phosphoamino-acid analysis of T 259 phosphorylated by cdc2. *c*, Proteolytic cleavage fragment phosphorylated by cdc2.

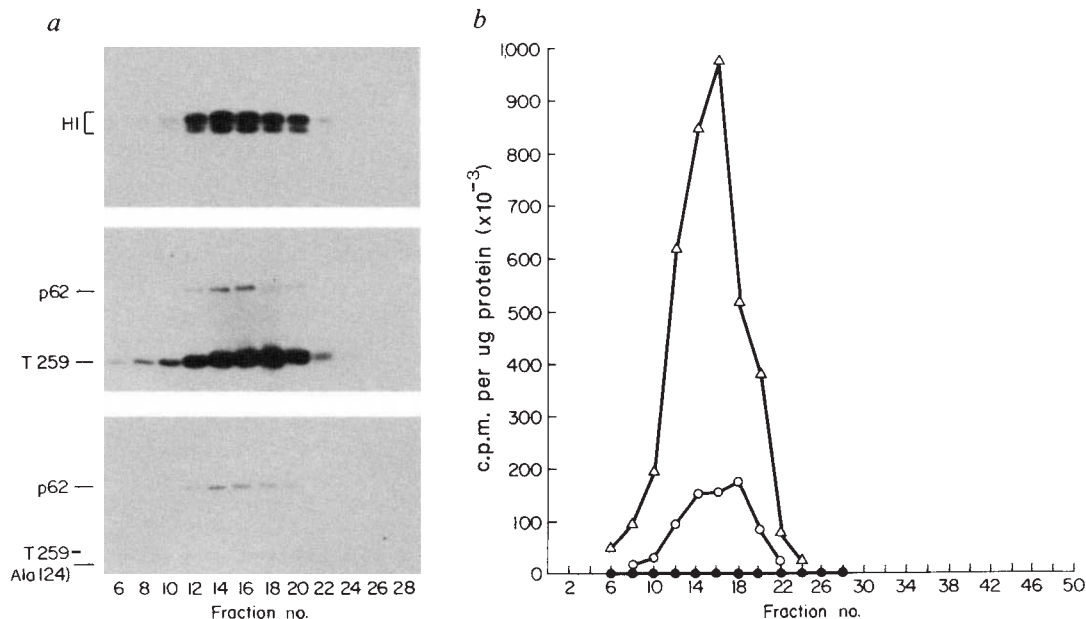
METHODS. The N-terminal 259 amino acids of SV40 large T antigen containing an extra 3 amino acids at the C-terminal end of the molecule was expressed and purified from *E. coli*³³. cdc2 protein kinase was purified as described previously³². T 259 (10 µg) incubated with 1–5 µl cdc2 for 60 min at 30 °C in 50 µl of 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM DTT, 50 µM ATP and 50 µCi [γ -³²P]ATP (3,000 Ci mmol⁻¹ (NEN)). A fraction of the reaction was separated in SDS-PAGE⁵² (10% acrylamide gel). The gel was stained with Coomassie blue and exposed for autoradiography. For the phosphoamino-acid analysis, half of a kinase reaction was precipitated with trichloroacetic acid and the protein was hydrolysed by HCl vapour, under N₂ atmosphere, at 105 °C for 90 min (according to PICO-TAG instructions). The hydrolysed amino-acids were resuspended in 10 µl of water containing 7 mg ml⁻¹ cold phosphoamino-acid standards and 2 µl were electrophoresed in two dimensions on TLC plates (250 µm Whatman K2 cellulose). The migration of unlabelled standards was determined by ninhydrin staining and labelled amino acids were detected by autoradiography. For proteolytic cleavage of phosphorylated T, analytical digests were performed on 1 µl of the phosphorylation reaction mixture. Preparative digestion was performed on 120 µl, reduced to 10 µl in a vacuum centrifuge (Savant). Samples were redissolved in 8 M urea, 0.4 M ammonium bicarbonate, and 4.5 mM dithiothreitol (OTT), and incubated at 50 °C for 15 min. Iodoacetic acid (5 mM) was added and alkylation was allowed to occur for 15 min at 37 °C. The reaction was terminated with an additional aliquot of dithiothreitol. The samples were diluted with a three-fold excess of water, resulting in a solution that was 2 M urea, 0.1 M ammonium bicarbonate. An aliquot (2 µl for analytical, 5 µl for preparative) of trypsin (EC 3.4.21.4; TPCK-treated, Worthington; 1 mg ml⁻¹ in 1 mM HCl) was added, and the digestion allowed to proceed for 12 h at 37 °C. A second aliquot was added, and the reaction incubated for an additional 12 h. Digestion with *S. aureus* (strain V8) protease (EC 3.4.21.19; Worthington; 1 mg ml⁻¹ in 0.1 M ammonium bicarbonate) was done as with trypsin. The preparative digest received three aliquots of protease over 24 h. Proteolytic digests were subject to electrophoresis on 16% (w:v) acrylamide gels in the presence of SDS and urea, using the Tris-tricine buffer system³⁴. Analytical digests were run on 1.0-mm thick gels, and preparative digests on 0.75-mm thick gels. After electrophoresis,



the gels were stained with Coomassie brilliant blue and exposed to autoradiography. On the preparative gels, the portions of the gel corresponding to the visible spots on the film (see panel c) were excised, and eluted overnight in buffer containing 6 M urea, 0.2 M ammonium bicarbonate, 0.1% (w:v) SDS. The eluate was dialysed using 1,000 nominal molecular mass cutoff membrane on a microdialysis apparatus (Pierce), and the sample was reduced in volume to near dryness in a vacuum centrifuge. The sample was suspended in 50 µl water and precipitated with 9 volumes of acetone for 60 h at -20 °C, and collected by centrifugation. The resulting pellet was redissolved in 0.1% trifluoroacetic acid, and subject to HPLC on a C₈ silica (300 pore size) column (2.1 × 50 mm, Applied Biosystems) using a Hewlett Packard 1090M chromatograph. The optical absorbance of the effluent was monitored at 214 nm. Peaks containing radioactivity were analysed by automated sequencing on an Applied Biosystems 470A instrument, in which 45% of the sample was injected on-line to an Applied Biosystems 120A Analyser.

FIG. 2 Comparison of histone H1 and T-antigen kinase activities in fractions of a Mono S column. *a*, Assay of the kinase activity cross the column using either histone H1 (top), T 259 (centre) or T 259-Ala 124 (bottom) as substrate. p62 is the endogenous cyclin-B substrate of the kinase. *b*, Quantitation of the labelling of histone H1 (triangle), T 259 (open circle) or T 259-Ala 124 (closed circles) in the kinase assays.

METHODS. Highly purified cdc2 kinase obtained from HeLa cells as described³² was rechromatographed using a Mono S 5/5 column (Pharmacia). Fractions from the column were assayed for kinase activity using 1 µg of either calf thymus histone H1 (Boehringer), T 259 or T 259-Ala 124. The mutant was obtained by oligonucleotide directed mutagenesis^{37,38} of T 259 to create a single amino-acid substitution consisting in a change of Thr 124 to Ala 124. The kinase reactions were carried out for 10 min at 30 °C in the following buffer conditions: 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM DTT and 5 µM ATP and 10 µCi [γ -³²P]ATP. The reactions were terminated by the addition of



electrophoresis sample buffer. A fraction of the stopped reactions were then fractionated on SDS-PAGE followed by Coomassie blue staining of the gels and autoradiography. Alternatively, the sample was precipitated with trichloroacetic acid (TCA) and acid insoluble counts were determined by autoradiography.

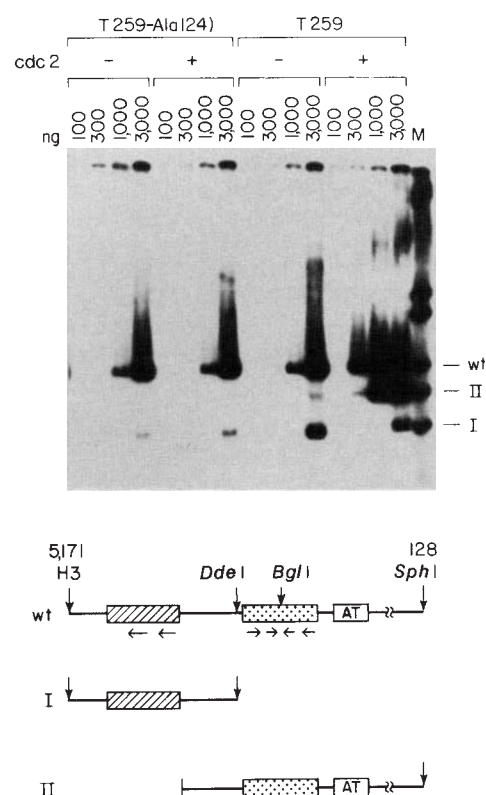
FIG. 3 DNA-binding properties of T 259 and T 259-Ala 124 exposed to cdc2. Top, indicated amounts of T, exposed (+) or unexposed (-) to cdc2 were incubated with an equi-molar mixture of end-labelled fragments shown in the marker lane (M). Protein-DNA complexes were precipitated with Pab419 and the bound DNA was analysed by SDS-PAGE. The migration positions of fragments bearing wild-type (wt), site 2 alone (II) or site 1 alone (I) are indicated. Bottom, maps of the DNA templates containing either site I, site II or the wild-type origin from SV40 used in the origin binding assays. The boxes represent the individual sites and the horizontal arrows represent the pentanucleotide recognition sites of T. Vertical arrows demark cleavage sites for restriction endonucleases.

METHODS. Parallel samples containing 6 µg of immunoaffinity purified T 259 or T 259-Ala 124 were preincubated in 10 µl of kinase buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl₂, 1 mM DTT) containing 75 µM ATP at 30 °C for 3 min. To half of the samples 1 µl cdc2 kinase was added and the reactions were incubated at 30 °C. After 1 h the reactions were placed on ice. Each titration point of T was brought to a final volume of 30 µl with kinase buffer containing 75 µM ATP. An equimolar mixture of DNA preparations carrying site I (pOS-1; SV40 nucleotides (nt) 5, 171-5,228), site II (pSV0d13; SV40 nt 5,209-128) and wild-type origin (pSV0⁺; SV40 nt, 5,171-128)^{15,33} were digested with *TacI*. The mixture was end-labelled with the large fragment of DNA polymerase I (Klenow) in the presence or radiolabelled deoxynucleotides, extracted with phenol and chloroform, and ethanol precipitated. This mixture (50 ng) in 30 µl of 2× origin binding buffer (20 mM HEPES, pH 7.4, 200 mM KCl, 2 mM MgCl₂, 10% glycerol, 100 µg µl⁻¹ BSA) was added to each 30 µl titration point of T (described above) and incubated on ice for 1 h. Anti-T monoclonal antibody (7.5 µg) (Pab 419) (ref. 40) in 5 µl of 1× origin binding buffer was added and allowed to incubate for a further hour. Protein A Sepharose slurry (10% (v/v), 100 µl in NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% NP40) was then added and the reactions were incubated on a rocker for 50 min at 4 °C. The beads were pelleted, washed three times with 1 ml of NET buffer, resuspended in 1% SDS, 25 mM EDTA, incubated at 65 °C for 15 min and electrophoresed on a 6% native polyacrylamide gel. Gels were exposed to Kodak XAR film.

properties (Fig. 4, note that the mammalian T 708 used in this study was purified from HeLa cells infected with an adenovirus construct carrying T 708, ref. 21).

DNA synthesis

Full-length T antigen purified from *E. coli* was used to investigate the effect of protein phosphorylation on the ability of T antigen



to initiate SV40 DNA synthesis. Underphosphorylated T from bacteria replicates SV40 DNA *in vitro* to only 10-15% of the level of replication directed by T antigen purified from HeLa cells²⁹. To assess the effect of cdc2 phosphorylation on the ability of T to initiate SV40 DNA synthesis it was first necessary to establish conditions for the kinase reaction that were compatible with subsequent assay of *in vitro* DNA replication^{14,20}. This was

essential because the ATP required as a source of phosphate in the kinase reaction acted as a potent inhibitor of subsequent DNA synthesis (data not shown). This may be due to ATP-dependent formation of higher order aggregates of T antigen^{41,42}. Using mammalian T 708 in test experiments, the inhibitory effect of ATP was overcome if the mock kinase reaction was performed in the presence of origin-containing template DNA. Levels of DNA replication obtained following preincubation in the presence of both ATP and DNA template, were similar thereafter to those directed by identical amounts of T 708 maintained on ice in the absence of ATP (data not shown). For this reason, in all further experiments the kinase reactions were performed in the presence of template DNA.

As described previously^{14,20}, T 708 purified from HeLa cells is highly active in directing DNA replication *in vitro*, whereas that from *E. coli* is relatively weak²⁹ (Fig. 5). Exposure of *E. coli*-derived T 708 to the Mono-S peak fraction of cdc2 kinase stimulated the ability of the protein to initiate SV40 DNA synthesis *in vitro* (Fig. 5). This effect was abolished if the kinase preparation was briefly boiled before being incubated with T, or if the template DNA did not contain the SV40 replication origin (Fig. 5). No further enhancement of DNA replication

was observed if HeLa cell purified T 708 was exposed to cdc2 *in vitro* (data not shown).

Discussion

The primary observation of this report is that phosphorylation of SV40 T antigen at Thr 124 by the cdc2 protein kinase increases the capacity of the protein to bind to the viral origin of replication and further to initiate DNA replication *in vitro*. Previous studies have shown that Thr 124 is phosphorylated *in vivo*²³ and that the integrity of this residue is essential for origin binding and DNA replication^{27,28}. No protein kinase capable of phosphorylating this site has previously been identified. Indeed, the present experiments provide the first direct demonstration that phosphorylation at Thr 124 modulates the sequence-specific DNA binding and replication activities of T.

Bacterial-derived T antigen has similar properties to mammalian protein that contains a single lesion at Thr 124 (refs. 27, 29). Neither polypeptide binds efficiently to site 2 of the viral origin. It is interesting, however, that the bacterial protein displays a weak ability to drive DNA replication, whereas the T-Ala 124 mutant protein purified from mammalian cells does not (I.M. and E. Fanning, unpublished results). It is possible

FIG. 4 DNA-binding properties of full length T 708 purified from either *E. coli* or human HeLa cells. Protein was either exposed to cdc2 (+) or not (-), before being assessed for DNA binding.

METHODS. Parallel sets of reactions containing 50, 150 and 450 ng of immunoaffinity purified *E. coli* and HeLa derived full length Tag (see fig. 5) in 20 μ l of 30 mM HEPES-KOH pH 7.4, 7 mM MgCl₂, 0.5 mM DTT containing 500 μ M ATP were assembled on ice. To one set of reactions an aliquot of highly purified cdc2 was added. All reactions were immediately incubated at 26 °C for 25 min. The reactions were removed and placed on ice. An equi-molar mixture (50 ng) of site I, II and wild-type origin-bearing fragments (Fig. 3) in 30 μ l of 1 $\times$ origin binding buffer (Fig. 3) was added to each tube. After incubating on ice for 50 min 5 μ l of 1 μ g ml⁻¹, Pab 419 (ref. 40) in 1 $\times$ origin binding buffer was placed in each reaction. A 50 min incubation on ice followed. The remainder of the experiment is as described in Fig. 3.

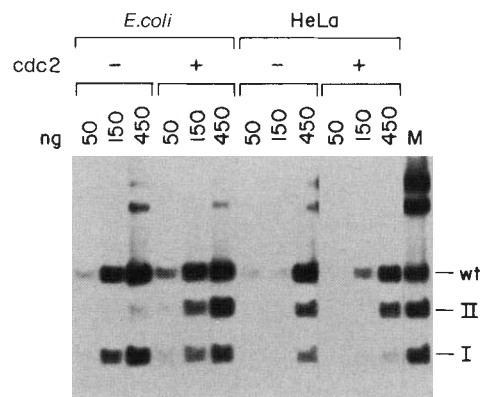
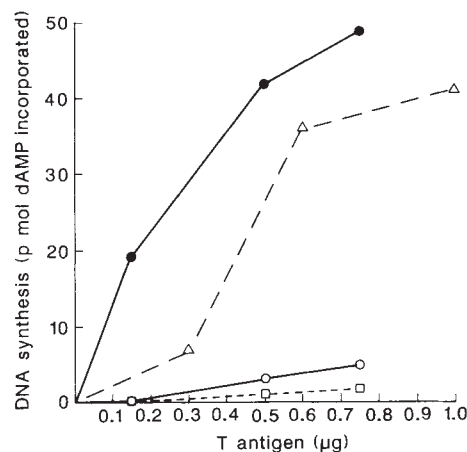


FIG. 5 Activation of T-dependent DNA replication by cdc2. DNA synthesis, assessed by incorporation of dAMP into origin-containing template DNA, was driven by indicated amounts of input T 708 from different sources. Bacterial T phosphorylated by cdc2 (closed circles), HeLa cell T (triangles), bacterial T exposed to boiled cdc2 (open circles), bacterial T exposed to native cdc2 (squares), but using template DNA lacking a viral replication origin.

METHODS. Full length T antigen from *E. coli* was purified by a modification of a published procedure (ref. 29). The high speed supernatant described was brought to 30% saturation with solid (NH₄)₂SO₄. The supernatant from this precipitation step was then brought to 55% saturation with solid (NH₄)₂SO₄. The 30–55% precipitate was then dissolved and dialysed against 1 litre of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol (2 changes of buffer, 500 ml, each for 1 hour). This fraction was then applied to an immunoaffinity column containing immobilized Pab 149 (ref. 40) as described previously²¹. The neutralized eluate containing peak fractions from this column were then loaded onto a 100 μ l double strand DNA cellulose column (Sigma) equilibrated in 20 mM HEPES-KOH, pH 7.4, 5% glycerol. Unbound material was removed by washing the column in this same buffer. The column was then washed with 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 5% glycerol. T antigen was eluted in 20 mM HEPES-KOH, pH 7.4, 400 mM NaCl, 5% glycerol. Peak fractions were pooled, dialysed overnight against 20 mM HEPES-KOH, pH 7.4, 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, snap frozen in a dry ice-ethanol bath and stored at -70 °C until use. All kinase reactions and DNA synthesis reactions were assembled on ice. 1 μ l of cdc2 kinase (mono S fraction) was added to reaction vessels containing various amounts of purified T antigen and 300 ng of either wild type plasmid or 8–4 DNA (ref. 12) in 30 mM HEPES, pH 7.4, 7 mM MgCl₂,



0.5 mM DTT, 0.5 mM ATP. The final reaction volume was 20 μ l. Kinase reactions were incubated at 26 °C for 25 minutes. The reactions were placed on ice whereupon they received a 30 μ l aliquot containing a cytosolic fraction from human 293 cells (S100), with the additional reagents necessary for SV40 DNA synthesis^{14,20}. After 2.5 hours at 37 °C, the products of the reaction were precipitated with trichloroacetic acid and collected by filtration onto glass fibre filters. The amount of acid insoluble radioactivity was quantitated by counting the dried filters in liquid scintillant.

that the wild-type bacterial protein becomes partially phosphorylated on Thr 124 during incubation in the cell-free lysate. Lysates prepared in a similar way to those used in this study, a crude S100 fraction of human 293 cells (Fig. 5, legend), contain cdc2 detectable by immunoblotting (B. Stillman, personal comm.).

It is striking that bacterial T 708 that has been modified by cdc2 *in vitro* is more active in DNA replication than the HeLa cell protein. Previous studies of T phosphorylation have identified an inhibitory class of serine phosphorylation sites^{21,22,26}. Exposure of T to calf intestinal alkaline phosphatase (CIAP), which dephosphorylates phosphoserine but not phosphothreonine, activates the ability of the protein to bind the origin and replicate DNA. This observation may also be related to the purification of type 2A phosphatase as a replication activating protein in T-dependent cell lysates⁴³. The cdc2 phosphorylated *E. coli* T 708 may resemble T antigen from mammalian cells which has been treated with CIAP or type 2A phosphatase.

It is of particular interest that cdc2 is able to phosphorylate T at the critical residue of Thr 124. In fission yeast, cdc2 function is essential both for the initiation of DNA replication and also, in association with the cdc13-encoded cyclin⁴⁴, for the entry in mitosis³¹. Much evidence supports the idea that in vertebrate cells the protein kinase also plays an essential role in regulating mitosis⁴⁵⁻⁴⁸ but the present experiments provide the first indication that cdc2 might also act in the initiation of DNA replication, albeit in a viral model.

In considering the physiological significance of our results the source of the cdc2 kinase should be considered. The enzyme used in the present study consists of cdc2 (p34) and cyclin B (p62) subunits and was purified in a mitotically activated state from cells arrested in M phase with nocodazole³². This enzyme is thought to represent the M-phase inducing factor (MPF), that drives an interphase cell into mitosis³⁶ and yet in the present experiments it is being used to promote T-dependent DNA replication.

It is possible that T becomes phosphorylated on residue Thr 124 by cdc2 during mitosis, but the available evidence indicates that passage of the host cell through M phase is not essential for viral replication. For example, CV1 cells that have been synchronized by mitotic shake-off can be infected with SV40 in the presence of hydroxyurea, which prevents DNA replication. Upon removal of the drug, viral replication proceeds at approximately the same time as cellular replication⁴⁹. In this

situation, T would not have encountered a mitotic form of cdc2 in the interval between infection and DNA replication, unless one property of the virus is to inappropriately induce the M phase form of cdc2. It is conceivable that this occurs and that cells fail to enter mitosis prematurely because cdc2 is diverted from its normal mitotic role into the phosphorylation of newly-synthesized T, which instead drives viral DNA replication.

Alternatively, the form of cdc2 used in the present study may not be that responsible for the phosphorylation of T at Thr 124 *in vivo*. The cdc2 molecule associates with proteins other than cyclin B. A complex between cdc2 and a polypeptide with $M_r \sim 60K$ (p60) has been described in HeLa cells⁵⁰. The identity of p60 is presently unknown, except that the same protein is found in association with E1A in adenovirus-infected cells. From the present perspective, the critical feature of the p60/cdc2 enzyme is that it phosphorylates histone-H1 *in vitro*, but is maximally active during interphase, whereas the cdc2/cyclin B enzyme used here is most active during mitosis^{45,50}. It is possible that p60/cdc2, or even cdc2 associated with other regulatory polypeptides, might be primarily responsible for the phosphorylation of T on residue Thr 124 at the initiation of viral replication.

SV40 encodes a T protein which requires phosphorylation on Thr 124 to initiate viral DNA synthesis. This phosphorylation event may represent a mechanism by which the virus senses the state of the cellular replication apparatus. Regulation of T by such a mechanism would prevent abortive initiation and premature template unwinding. The replication machinery in quiescent cells is dormant, thus preventing viral DNA synthesis even if active T was present. In this scheme, T produced at early times after infection is not phosphorylated at Thr 124 and could not initiate viral DNA replication. It does, however, stimulate cellular DNA synthesis⁴⁹. An induced S-phase kinase (containing cdc2) could then phosphorylate Thr 124 and serve as a positive molecular switch by activating viral replication. Consistent with this hypothesis, a form of T which binds site-I DNA has been detected 8 h before a site-II binding form in productively infected cells⁵¹.

The present experiments provide no direct information about a possible role for cdc2 in the initiation of cellular DNA replication. A protein or complex of cellular proteins, however, must perform the origin binding role that T serves in viral DNA replication. The phosphorylation and activation of such replication elements by cdc2 might be a critical step in the initiation of cellular DNA synthesis. □

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A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*

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The patterning of cells in insect segments requires the exchange of information between cells, which in *Drosophila* depends on the activity of members of the segment-polarity class of genes. Here we report the molecular characterization of one such gene, *patched*. We find that *patched* encodes a large protein with several possible membrane-spanning domains and is expressed in a complex pattern during embryogenesis.

DURING the earliest stages of *Drosophila* embryogenesis a series of positional cues are established along the antero-posterior axis of the embryo (reviewed in refs 1–3). These cues, which correspond to the expression domains of the pair-rule genes, allocate the newly forming cells to individual parasegmental primordia^{4,5}. Although much is now known about the intracellular signals that establish the pair-rule gene-expression domains³, the way in which the information that they encode is transformed to give the detailed pattern of each larval body segment remains poorly understood.

Generation of the segment patterns depends on the activity of the segment-polarity class of genes⁶, mutations of which cause locus-specific deletions of different parts of each segment, accompanied by the duplication of the remaining structures. In the absence of expression of the *patched* (*ptc*) gene⁶ the middle region of each segment is deleted (Fig. 1); by contrast, mutations at several other loci cause the deletion of the posterior part of each segment. Several of these latter genes have been cloned, and two, *engrailed* (*en*)^{7,8} and *gooseberry* (*gsb*)^{9,10} have been found to encode homeodomain-containing proteins. Both of these genes, together with a third member of the class, *wingless* (*wg*)¹¹, are expressed in specific domains in each parasegment during most of embryogenesis^{8–12}; these patterns of expression are first established as the blastoderm cellularizes in response to different combinations and levels of pair-rule gene

activity^{4,13–15}. These early defined domains mark the boundaries of the parasegmental primordia and can be regarded as providing the reference points for the subsequent elaboration of positional information in each parasegment.

In contrast to the positional signalling that occurs before cellularization, patterning in parasegments seems to involve signalling between cells during the proliferative phase which follows cellularization. The best evidence for this comes from studies of the segment polarity genes themselves. The continued expression of *en*, for instance, requires the expression of *wg* in neighbouring cells^{16,17}, implying that *wg* is involved in the production of a stimulatory signal that is transmitted to the *en*-expressing cells^{16,17}. This interpretation is strongly supported by the extra-cellular distribution of the *wg*-encoded protein¹⁸ and is consistent with the known properties of the growth-factor-like product of the mouse proto-oncogene *int-1*, to which *wg* is closely related¹⁹.

Whereas in normal embryos only those cells posterior to each *wg*-expression domain initiate and maintain *en* expression, in *ptc* mutant embryos, *en* expression is additionally induced in cells anterior to each *wg*-expression domain^{16,17}. This indicates that one of the normal functions of the *ptc* gene is to determine the competence of cells to respond to the *wg* signal and hence maintain the appropriate polarity of each parasegment. To investigate the molecular basis of this phenomenon, we have cloned the *ptc* gene. Here we describe its pattern of expression during embryogenesis and show that it encodes an integral membrane protein with multiple transmembrane domains, a structure which is characteristic of several proteins, including membrane-associated receptors.

Molecular cloning of *patched*

To clone the *ptc* gene we induced new alleles of the locus by hybrid dysgenesis and isolated recombinant phage with P-element homology from a genomic library constructed from one such allele. By using fragments from this clone as probes, we cloned and mapped 40 kilobases (kb) of contiguous wild-type DNA (see Fig. 2a legend for details). Transcriptional analyses of the region showed that it contains a single transcription unit extending over ~17 kb and encodes a transcript of ~5.8 kb which is expressed during most of embryogenesis (data not

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FIG. 1 a, Ventral cuticle of the fourth and fifth abdominal segments of a wild-type first-instar larva. Note the distinctive pattern of denticles, each row being composed of elements of characteristic size and polarity. The arrowheads indicate the region of the segmental pattern deleted in *ptc* mutants. b, The equivalent region of a larva homozygous for a deficiency of the *patched* gene *ptc*^{G12}. In contrast to that in wild-type larvae, the denticle belt is mirror symmetric, the posterior part of each denticle belt being replaced by an anterior part with reverse orientation.

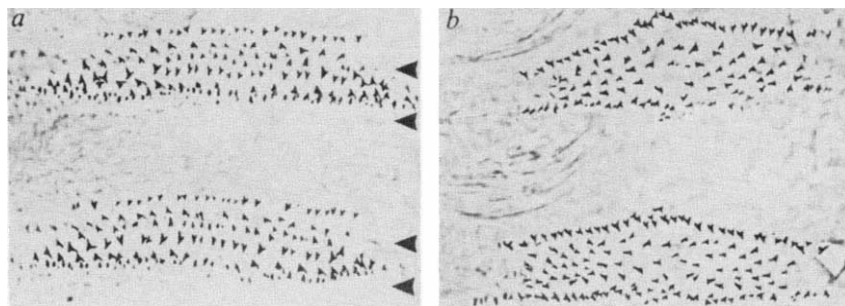
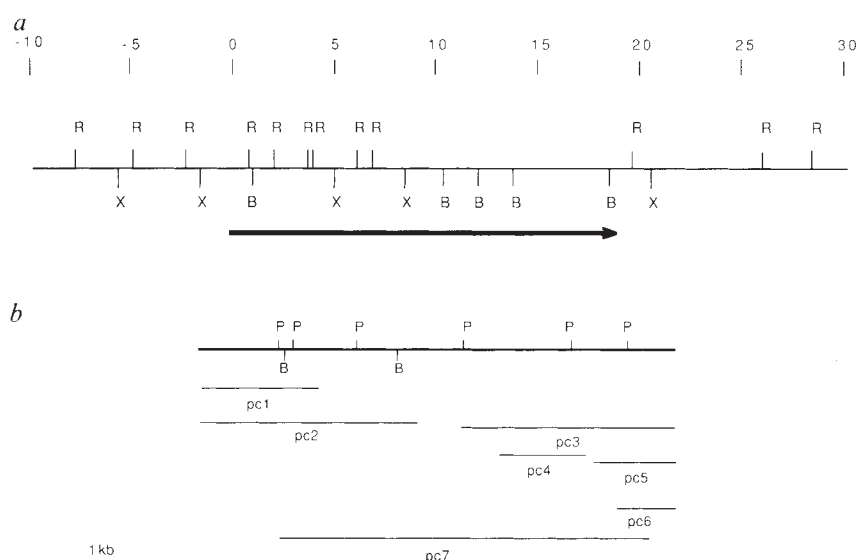


FIG. 2 *a*, Restriction map of the genomic DNA corresponding to the *ptc* gene. The solid line indicates the transcribed region; position 0 is the approximate location of transcription initiation. *b*, Restriction map of the cDNA clones corresponding to the *ptc* transcription unit. B, *Bam*HI; R, *Eco*RI; P, *Pvu*II; X, *Xho*I.

METHODS. Eight new *ptc* alleles were isolated from among 250,000 flies after hybrid dysgenesis. By *in situ* hybridization, four of these were found to be associated with P-element insertions at the region 44D3, 4 on the right arm of chromosome 2 in the cytological region to which *ptc* had been mapped⁴⁰. One of these alleles, *ptc*^{P78}, was recombined onto a P-element-free second chromosome, and DNA from these flies was extracted, partially digested with *Mbo*I and cloned into the λ -phage vector EMBL-4 (Stratagene). Recombinant phage with P-element homology were isolated by plaque hybridization and clones deriving from the 44D3, 4 region were identified by *in situ* hybridization to P-element-free polytene chromosomes. By using the genomic sequences flanking the P-element insert as probes, we isolated 40 kb of contiguous DNA from wild-type genomic libraries. Comparison of the wild-type map with that of the mutant clone shows the mutant clone has a deletion of ~15 kb of wild-type sequences, together with an insertion of a P element (not shown). To confirm that the *ptc*^{P78} allele is indeed associated with this deletion, DNA from flies heterozygous for the mutation was digested with *Eco*RI and probed with fragments that include the deletion breakpoints. Novel bands are seen with both probes, consistent with the deleted structure predicted from the clone. Southern transfer analysis of 16 other *ptc* mutations showed that 8 of them have alterations in the restriction map of the cloned region (I.G., A.H., Y.N., R. Phillips and P.W.I., manuscript in preparation). To identify transcribed regions of the cloned DNA, asymmetric ³²S-labelled RNA probes were prepared by *in vitro* transcription of each of the *Eco*RI fragments (subcloned in Bluescript



shown). Several complementary DNA clones derived from this transcription unit were isolated (Fig. 2*b*) and used to prepare probes for analysis by *in situ* hybridization.

Embryonic transcription pattern of *patched*

The pattern of expression of *ptc* is considerably more complex and dynamic than that of *en* (ref. 8), *gsb* (refs 9, 10) or *wg* (ref. 11). In contrast to *en*, *gsb* or *wg* transcripts, when *ptc* transcripts are first expressed in the stage-5 (cellularizing blastoderm) embryo, they are almost uniformly distributed around the periphery of the embryo (compare Fig. 3*a* and *b*). As gastrulation begins, a modulation of *ptc* transcript expression becomes apparent, and by stage 10 (when the germ band is fully extended), the transcript is expressed in broad bands in both the ectoderm and mesoderm of each parasegment, in contrast to the narrow stripes typical of the expression domains of *en* and *wg* at the same stage (Fig. 3*c, d*; Fig. 4). Comparison of adjacent sections of the same embryo hybridized with different probes shows that at this stage the *ptc* expression domain is complementary to that of *en* and overlaps that of *wg* (Fig. 4). In the mesoderm, expression is out of register with the overlying ectoderm, so that cells that still express *en* also express *ptc* (Fig. 4).

In stage 12 embryos, before the onset of germ-band shortening, a further restriction occurs in the ectodermal domains; the *ptc* transcripts disappear from the central region of each domain so that there are two narrow stripes of *ptc* expression in each segment, one marking the anterior boundary of each segment and the other overlapping the *wg*-expressing cells (Fig. 3*e-h*). This pattern of expression persists until the end of embryogenesis.

Predicted protein structure

The entire coding sequence of the *ptc* transcription unit was determined by dideoxy sequencing²⁰ of the cDNA clones.

vector) and hybridized to sections of 0–20-hour-old embryos. This analysis identified sequences within a region from position –2.5 to +20 that were transcribed both in the same direction and with identical spatial and temporal patterns during embryogenesis. Restriction fragments from each end of the transcribed region were used to screen an embryonic cDNA library⁷ and two sets of overlapping clones were isolated. Single-stranded probes prepared from the two longest non-overlapping clones (pc2 and pc3) were hybridized to northern-transfer filters of staged total RNA. Both probes detect a single transcript of ~5.8 kb with an identical expression profile; this transcript is most abundant in 6–12-h embryos but is present also in post-embryonic stages (data not shown). Clone pc7 was isolated by R. Phillips (personal communication) from a library constructed by N. Brown (unpublished results).

Sequence analysis of genomic subclones has revealed a consensus transcription initiation site and a possible TATA-sequence box just upstream of the 5' end of the pc2 cDNA clone (Y.N., unpublished observations), indicating that this represents the 5' terminus of the *ptc* messenger RNA.

By aligning the sequences of several overlapping cDNAs, we defined a contiguous stretch of 5547 nucleotides between the 5' end of pc2 and the 3' end of pc3, which agrees well with the size (~5, 8 kb) of the *ptc* transcript determined by northern-blot analysis (I.G., unpublished data). Conceptual translation of the complete cDNA sequence in all three reading frames reveals a single long open-reading-frame (ORF) of 3,897 nucleotides, preceded by a long 5' untranslated leader sequence of ~750 nucleotides. The context of the initiation codon AAC-CATAATGGAC (Fig. 5) shows a reasonable similarity to the consensus insect translational initiation sequence²¹. Downstream of the translation termination codon is an untranslated region of ~890 nucleotides. This region includes two potential polyadenylation signals having the canonical sequence AATAAA (ref. 22; Fig. 5). A third, atypical sequence, ATATAA, which could also function, albeit inefficiently, as a polyadenylation signal (N. Proudfoot, personal communication), is located 21 nucleotides from the 3' end of the pc7 clone. The 3' untranslated region is also characterized by five pentanucleotide repeats that have the sequence ATTGA (Fig. 5). These repeats are found in the 3' trailers of several genes including *c-fos* (ref. 23) and lymphokine granulocyte-macrophage colony-stimulating factor (GM-CSF)²⁴, where they confer message instability. We have not determined the complete intron-exon structure of the *ptc* gene, but comparison of the cDNA and genomic maps indicates a large 5' intron of at least 8 kb and several smaller introns at the 3' end of the transcription unit (data not shown).

The large ORF is capable of encoding a 1,299-amino-acid protein of predicted relative molecular mass (M_r) 144,000.

Hydropathy analysis²⁵ of the amino-acid sequence enabled us to predict the presence of seven main hydrophobic stretches, but no signal peptide sequence (Fig. 6). The lengths of three of these (A, B and E) are consistent with their spanning the membrane once, whereas regions C, D, F and G are large enough to cross the membrane twice or even three times.

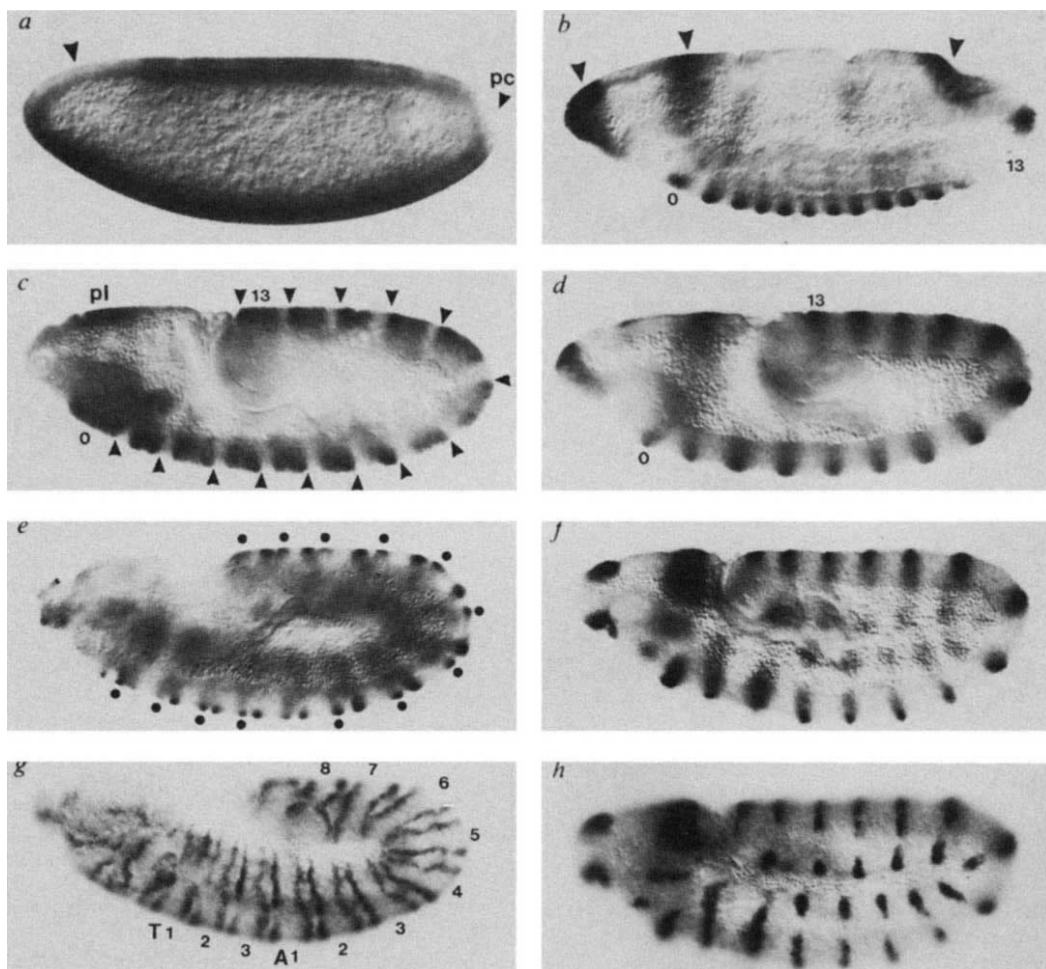
By considering the distribution of charged residues around and in these hydrophobic blocks, we have tentatively designated ten distinct transmembrane domains to the *ptc* gene product (Figs 5 and 6); two of these domains are long enough to cross the membrane twice, and hence enter and leave the membrane on the same side, in a manner analogous to that suggested for domain A of the CD20 antigen protein²⁶. The orientation of the Ptc protein relative to the membrane is based partly on the preferential location of clusters of positively charged residues on the inside of the membrane, but principally on maximizing the number of possible glycosylation sites located extracellularly. Of the eight possible glycosylation sites, six are located in the two large internal hydrophilic regions, which are accordingly positioned extracellularly in our model (Fig. 6). This configuration places both the amino terminus and carboxyl

terminus (which also contains two possible glycosylation sites) inside the cell, but other models, which position the C-terminus outside the cell, are also possible (not shown). The C-terminal region is highly charged and is characterized by a high proportion of proline and serine residues; the serines are potential targets for kinases.

Discussion

As a member of the segment-polarity class of genes, *ptc* has a key role in the generation of the normal pattern in each segment in *Drosophila*. Three of these genes, *en*, *gsb* and *wg*, are expressed in restricted domains of each segment; their expression patterns are first established at the blastoderm stage of embryogenesis in response to the activities of pair-rule genes. These segment-polarity genes therefore seem to function by registering positional cues generated by pair-rule genes and maintaining this positional specification during subsequent development. Although they are expressed at the boundaries of each parasegment^{5,11,13}, it has been suggested that pair-rule genes have the capacity to specify different positional values throughout each parasegment primordium. This could be achieved either by

FIG. 3 Comparison of the spatial distribution of *ptc* and *wg* transcripts during embryogenesis. *a*, In the cellularizing blastoderm embryo (stage 5), *ptc* transcripts are almost uniformly distributed, except for an antero-dorsal region (arrow head), where levels are consistently lower and in the pole cells (PC) where no transcripts are detectable. Transcripts are also detectable in the internal yolk nuclei, although they are not visible in this preparation. This pattern contrasts with that of *wg* transcripts *b*, which are initially expressed in three distinct domains (indicated by arrowheads) and which by stage 6 (shown here) are expressed in a series of stripes that mark the posterior boundaries of parasegments 0–13. *c*, After gastrulation, *ptc* expression becomes spatially restricted; in the stage-10 embryo, it is expressed in the posterior three-quarters of the ectoderm of each of parasegments 0–13 (the boundaries of which are indicated by arrowheads). This expression domain is complementary to that of *en* but overlaps the *wg*-expression domains shown in *d*. At this stage, significant expression of *ptc* is also detectable dorsally in the procephalic lobe (PL) in a region bounded by the two anterior domains of *wg* expression. *e*, Towards the end of the extended germ-band stage (stage 12), each band of *ptc* expression in the ectoderm splits in two, with the anterior stripe, which marks the anterior boundary of each incipient segment, showing stronger *ptc* expression than the posterior stripe. The latter, indicated by black dots, coincide with the stripes of *wg* expression shown in *f*. Whereas by this stage most of the *wg*-expression stripes have split into a dorsal and a ventral component⁴¹ *h* the *ptc*-expression stripes *g* remain continuous across the width of each segment (T1–T3, thoracic; A1–8, abdominal). All photographs show embryos oriented anterior to the left, dorsal surface uppermost. Staging is according to Campos-Ortega and Hartenstein⁴².



tated anterior to the left, dorsal surface uppermost. Staging is according to Campos-Ortega and Hartenstein⁴².

METHODS. Embryos were fixed as described⁴³ and hybridized with probes prepared from cDNA clones *pc2* (*ptc*) or *D-int1* (*wg*)¹⁹ labelled with digoxigenin (Boehringer) according to a protocol devised by D. Tautz and C. Pfeifle, modified by C. Oh and B. Edgar (personal communication). Signal detection was as described by the manufacturers; embryos were mounted in JB4 (Polysciences) and photographed using Direct Interference Contrast optics on a Zeiss Axioplan microscope.

means of a graded distribution of protein products of the pair-rule genes²⁷, or through their combinatorial activity in different cells²⁸. An implication of these models is that each band of cells in a primordium might register its position by the pair-rule-dependent activation of different segment-polarity genes. In such models, *ptc* would seem a good candidate for spatially regulated activation, because it is the only zygotically expressed segment-polarity gene known to be required for the specification of pattern in the middle region of each segment. We find, however, that at the blastoderm stage, *ptc* expression is almost uniform, its pattern only becoming spatially restricted during germ-band extension. These findings not only argue against a role for *ptc* in allocating specific blastoderm cells to particular pathways of development, but also support the view that such specification is limited to the boundary cells of each primordium³.

By analysing the sequence of the *ptc* transcript we predicted that it encodes a protein with a structure that is novel among the products of segment-polarity genes^{7-9,19,29}. The most striking feature is the presence of several hydrophobic domains, which have the potential to span the plasma membrane. Multiple membrane-spanning domains are a feature of several previously described proteins, which can be classified into two groups. The first includes the mating pheromone receptors of the yeast *Saccharomyces cerevisiae*³⁰, the cyclic AMP receptor of the slime mould *Dictyostelium discoideum*³¹, as well as the adrenergic³²,

muscarinic acetylcholine³³ and serotonin³⁴ receptors in vertebrates. All these proteins have seven transmembrane domains and are coupled to second messengers by means of G proteins. The second group is more diverse, both structurally and functionally, and consists of proteins involved in transport across membranes, such as the erythrocyte band-3 protein³⁵, the Na⁺/H⁺ antiporter³⁶, the glucose transporter³⁷, and the P glycoprotein^{38,39}. In our tentative model of the tertiary structure of the *ptc* gene product, the protein spans the membrane twelve times, has two large potentially glycosylated extracellular domains and intracellular N- and C-terminal ends. This structure differs significantly from that of the G-protein-coupled receptor proteins; in addition, searches of the National Biomedical Research Foundation databases have revealed no significant homology between the *ptc* protein and any other previously described. Thus we conclude that *ptc* encodes a novel form of integral membrane protein.

Genetic analysis of *ptc* has implicated it in the regulation of the expression of both *wg* and *en*. The regulation of *en* expression, at least, depends on local cell interactions^{16,17}. In the absence of *ptc* expression, cells anterior to those expressing *wg* in each parasegment apparently respond inappropriately to the intercellular signal encoded by *wg*, and initiate expression of *en*. This indicates that one of the functions of *ptc* could be to modulate the competence of cells to respond to the *wg*-encoded signal^{3,16}. This role is consistent with the pattern of *ptc*

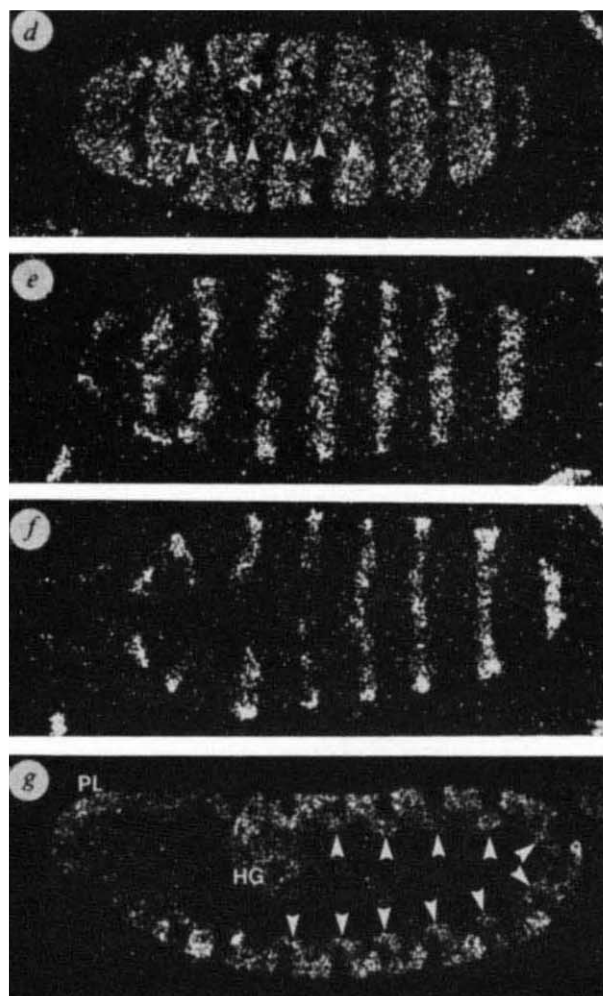
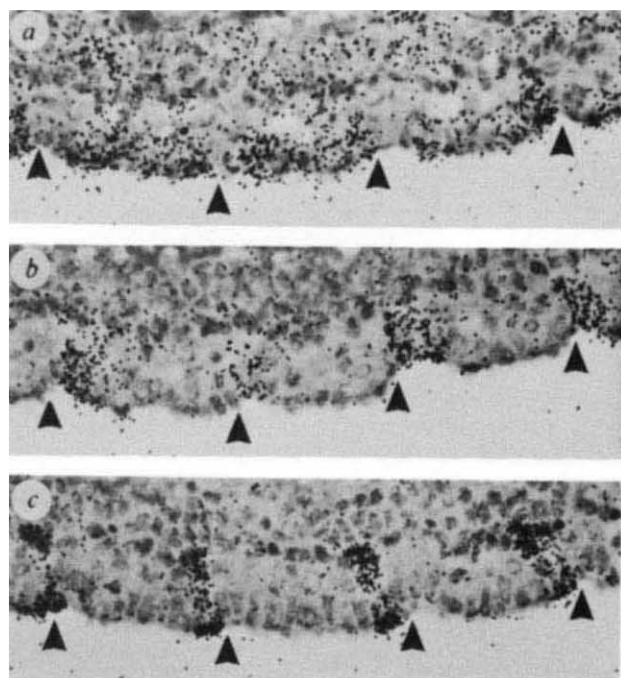


FIG. 4 Comparison of the *ptc*, *en* and *wg* expression domains. *a-c*, Details of parasegments 4-6 in adjacent sections of a stage-10 embryo hybridized with probes for *ptc* (*a*), *en* (*b*), and *wg* (*c*) labelled with tritium and visualized by autoradiography. The parasegmental boundaries are indicated by arrowheads. Note that *ptc* transcript is not detectable in cells that express *en*, but is present in the rest of each parasegment, including those cells that express *wg*. *d-f*, Dark-field images of adjacent frontal sections through the ventral region of a stage-10 embryo hybridized with probes for *ptc* (*d*), *en* (*e*), and *wg* (*f*). Note the greater width and the phase shift of the ectodermal and mesodermal (arrowheads) domains of *ptc* expression relative to the narrower, continuous bands of *en* and *wg* expression. *g*, Dark-field image of a section through a stage-10 embryo hybridized with tritiated *ptc* probe. This shows that the bands of expression are out of register in the mesoderm (arrows) and ectoderm. Note also the expression in the hindgut (HG) and the procephalic lobe (PL).

METHODS. Wax-embedded tissue sections were prepared and hybridized

with tritiated single-stranded RNA probes essentially as previously described⁴³. Probes were synthesized from cDNA clones pc2 (*ptc*), pwg-c14 (*wg*)¹¹ and E2C (*en*)⁷.

5'ACAGATACATCTCCAGACTCGCTGGGATCTCCGAAGGAAA	41	TTT CAG CAC TAC ACT CCC TTC CTC ATC CGC AGC TGG GTG AAG TTC CTC ACC GTT	2803		
CGGTGTGAAGTCGGAGCGCGACGACTGTGTTATCTGGTATTTCGCACTACTCGCACTCTCTGTGTGTGTAT	112	Phe Gln His Tyr Thr Pro Phe Leu MET Arg Ser Trp Val Leu Met Arg Leu Thr Val			
ACTAACACAGATAGATACACAGAAGCTGGTGAAAGGCTAAGATATTGTACCTCAGCAGTGGCAGGCGAAGT	183	ATG GGT TTC CTC GGG GGC CCC CTC ATA TCC AGC TTG TAT GGC TCC ACC GGC CTT CAG	2857		
TCATGGATTAAATGCCAGCAACAAACAAAGCCAGCAGCAGCAGCTGTTGTGTGTGCTGCCCAAG	254	MET Gly Phe Leu Ala Leu Ala Leu Ser Leu Thr Arg Leu Gln			
TCGAAAGTAAAGATGGATGAAAGCGCAAGAGCGGAGAGAAAGCAAGCACTGGTGGCTCCGCACTGCC	325	VI			
CGTTTTCATGCTGTAAAGATCTGTGAAATTTCTGTCAAAATCCCTCAGAAATTTGGCCCAAGATAAAAC	396	GAT GGC CTC CAG ATT ATT GAT CTG GTG CCC AAG CAG AGC AAC GAG CAC AAG TTC	2911		
CGCAAAACCGCGTTTAAATCGTGGAAAAACCCAGAAACCGAGGACGAATCAACAAACAAACACACA	467	CTG GAT GCT CAA ACT CGG CTC TTT GGC TTC TAC AGC ATG TAT GCG GTT ACC CAG	2965		
ACGAGAGCTCAGATACACGCGTCTCAGTCACTGAGCAGAGAGCGCGGAGAGAGCGCTCTCTGTATT	538	Gly Asp Ala Gln Thr Arg Leu Phe Gln Thr Ser Leu Tyr Ala Thr Arg Leu Thr Gln			
AAATACAAAATAATAAAAATAAAATGGGAGTGCAGTGCAGAAATCAGCAACAAATAACGAGATGCC	609	GGC AAN TTT GAA TAT CCC ACC CAG CAG CAG CTG CTC AGG GAG TAC CAT GAT TCC	3019		
AATACCAATTATCGAACCGAAATCCAGCAACATCCGACACTGTCTCCCAAGTCTCAGTCTCTCGAGC	680	Gly Aan Phe Glu Tyr Pro Thr Gln Gln Gln Leu Asp Arg Asp Tyr His Asp Ser			
CGCAGCAGCTCCCATCTGCACATCTCCCGCAGCGATTCTCGTCCGGAATCTGGGTATAAACAATACCAT	751	TTT GTG CGG GTG CCA CAT GAT GTG ATC AAG AAT GAT AAT GGT GGA CTG CCG GAC TTC	3073		
ATG GAG CGC CAG AGC CTC CCA CGG GTT CCG GAG ACA CAG GGC GAT GTG GTC GAT	805	TGG CTG CTG CTC TTC AGC GAG TGG CTG GGT AAT CTG CAA AAG ATA TTC CAG GAG	3127		
MET Asp Arg Asp Ser Leu Pro Arg Val Pro Asp Thr His Gly Asp Val Val Asp		GAA TAC Arg CAG GGA GCG CTC ACC AAG GAG TCG TGG TCC CCA AAC GCG AGC	3181		
GAG AAA TTA TTC TCG GAT CTT TAC ATA CGC ACC AGC TGG GTG GAC GCC CAA GTG	859	Glu Tyr Cys Arg Asp Leu Phe Ser Leu Thr Ile Arg Thr Ser Trp Val Asp Ala Gln Val		GAT GCC ATC CTG GCG TAC AAG CTA ATC GTG CAA ACC GGC CAT GTG CAG AAC CCC	3235
Glu Lys Leu Phe Ser Asp Leu Phe Ser Leu Thr Ile Arg Thr Ser Trp Val Asp Ala Gln Val		CTG GAC AAG GAA CTG GTG CTC ACC AAT GGT CTC GTC AAC AGC GAT GGC ATC ATC	3289		
GCG CTC GAT CAG ATA GAT AAG GGC AAA CGC GGT GGC AGC CGC AGC GGC ATC TAT	913	Val Asp Lys Glu Leu Val Leu Thr Asn Arg Leu Val Asn Ser Asp Gly Ile Ile		AAC CAA CGC GGC TTC TAC AAT TAT CTG TCG GGA TGG GCG ACC AAC GCG TCT	3343
CTG CGA TCA GTA TTC CAG TCC CAG CTC GAA ACC CTC GGC AGC TCC GTG CAA AAG	967	Asn Gln Thr Arg Gln Thr Tyr Lys Leu Ile Val Gln Thr His Gly Val His Asp Pro		CCT ACG GAG CTT CTC AGG GCA AAT TGT ATC CGG AAC GCG GCC AAC GGA GGT TCT	3397
Leu Arg Ser Val Phe Gln Ser Leu Thr Glu Thr Lys Gly Ser Ser Val Gln Lys		CAG GGC AAA TTG TAT CGG GAA CGC GCG CAG TAT TTT CAA CAA CCG AAC GAG TAC	3451		
CAC GCG GGC AAG GTG CTA TTC GTG GCT ATC CTG GTG CTC AGC ACC TTC TGC GTC	1021	Gln Gly Lys Leu Tyr Pro Glu Pro Arg Gln Tyr Phe His Gln Pro Asn Glu Tyr		GAT CTT AAG ATA CCC AAG AGT CTG CCA TTG GCT TAC GAT AGT CCC TTT TAC	3505
His Ala Gly Lys Val Leu Phe Val Ala Ile Gly Val Leu Val Leu Thr Phe Cys Val		Asp Lys Lys Ile Pro Lys Ser Leu Thr Pro Leu Val Tyr Ala Thr Gln MET Pro Phe Tyr		CTC CAG GGA CTA ACA SMT ACC TCG CAG ATC AAG ACC CTC ATA GGT CAT ATT CCG	3559
CGC AAG AGC CGC CAG ATC CAG TCC AAG GTG CAG CAG CTC TGG ATC CAG GAG	1075	CTC ACG GAG CTT CTC AGG GCA AAT TGT ATC CGG AAC GCG GCC AAC GGA GGT TCT	3397		
Gly GCG GGG CTG GAG GAG GAA CTG GCG TAC ACA CAG AAG ACC ATC GGC GAG GAC	1129	CAG GGC AAA TTG TAT CGG GAA CGC GCG CAG TAT TTT CAA CAA CCG AAC GAG TAC	3451		
Gly Gly Gly Leu Glu Glu Glu Glu Ala Tyr Thr Gln Lys Thr Ile Gly Glu Asp		GAT CTT AAG ATA CCC AAG AGT CTG CCA TTG GCT TAC GAT AGT CCC TTT TAC	3505		
GAG TCG GCC ACG CAT CAG CTG CTC ATT CAG ACG ACC CAG CAG CCG AAC GCG TCC	1183	Asp Lys Lys Ile Pro Lys Ser Leu Thr Pro Leu Val Tyr Ala Thr Gln MET Pro Phe Tyr		CTC CAG GGA CTA ACA SMT ACC TCG CAG ATC AAG ACC CTC ATA GGT CAT ATT CCG	3559
Glu Ser Ala Thr His Gln Leu Glu Thr Ile Gln Thr His Asp Pro Asn Ala Ser		GAC CTG AGC CTC AAG TAC CAG GGC TTT GGC CTC CCG AAC TAT CCA TCG GGC ATT	3613		
CTG CTG CAT CCG CAG GCG CTC CTT GGC CAG CTG GAG CTC CTG CTC AAG GCG ACC	1237	Asp Leu Ser Val Lys Tyr Glu Gly Phe Gly Leu Pro Asn Tyr Pro Ser Gly Ile		CGC TTC ATC TTC TGG CAG CAG TAC ATC ACC CTC CCG TCC TCA CTG GCG ATT	3667
Val Leu His Pro Gln Ala Leu Leu Ala His Leu Glu Val Leu Val Lys Ala Thr		Pro Phe Ile Phe Trp Thr Thr Thr MET Thr Lys Arg Ser Ser Leu Met Thr Ile		CTG GCG TGC GTG CTA CTC GCG GCG CTG GTG CTC GTC TCC CTC CTC CTC TCC	3721
GCG GTC AAG GTG CAC CTC TAC GAG ACC GAA TGG GGG CTG GCG CAG ATG TGC AAC	1291	CTT GGA GCG Val Leu Leu Ala Leu Ala Val Leu Val Ser Leu Val Leu Gln Leu Ser		GTT TGG GCG GCG GTT CTC GTG ATC CTC AGC GGT CTC GCG TCG CTC GCG CAG ATC	3775
Ala Val Lys Val His Leu Tyr Asp Thr Glu Trp Glu Tyr Arg Asp MET Cys Asn		Val Trp Ala Ala Val Leu Val Ile Leu Ser Val Leu Ala Ser Leu Ala Gln Ile		TTT GGG GCG ATC ACT CTC GTG GGC ATC AAA CTC TCG GCG ATT CCG GCA GTC ATA	3829
ATG CCG AGC ACG CCC TCC TCG GAG GGC ATC TAC TAC ATC GAG CAG ATC CTG CCG	1345	CTC ATC CTC AGC GTG GGC ATG ATG CTG TGC TTC AAT GTG CTC ATA TCA CTG GCG	3883		
MET Pro Ser Thr Pro Ser Phe Gly Gly Ile Tyr Gln Thr His Asp Pro Asn Ala Ser		Leu Ile Leu Ser Val Gly MET MET Leu Cys Phe Asn Val Leu Ile Ser Leu Gly		CTC ATC CTC AGC GTG GGC ATG ATG CTG TGC TTC AAT GTG CTC ATA TCA CTG GCG	3883
CAC CTC ATT CCG TCG TCG ATC AGC CCG CTG GAG TGT TTT TCG GAG GGA AGC	1399	Leu Ile Leu Ser Val Gly MET MET Leu Cys Phe Asn Val Leu Ile Ser Leu			

FIG. 5 Nucleotide sequence of the *ptc* gene and predicted amino-acid sequences. Canonical polyadenylation signal (AATAAA), atypical polyadenylation signal (ATATAA) (see text) and mRNA destabilizing ATTTA motifs are underlined. The amino-acid sequence of the longest ORF is shown. Amino-acid sequences underlined correspond to predicted hydrophobic segments (I–X). Clusters of charged residues that are believed to mark the boundaries of membrane-spanning segments are shown in italics. Possible N-glycosylation sites (consensus: Asn-X-(Ser/Thr)) are boxed.

METHODS. The cDNA clones described in Fig. 2 were sequenced by the dideoxy technique²⁰ with Sequenase (US Biochemical). Both nested deletions, generated by *ExoIII* and *S1*-nuclease digestion and oligonucleotides corresponding to previously determined sequences were used in the sequencing reactions. The cDNA clone pc2 corresponds to nucleotides 1–2,583 and pc3 to nucleotides 3,028–5,547. Both strands were sequenced, excluding parts of the untranslated regions between nucleotides 1–213, 426–600, 4,833–4,921 and 5,302–5,547.

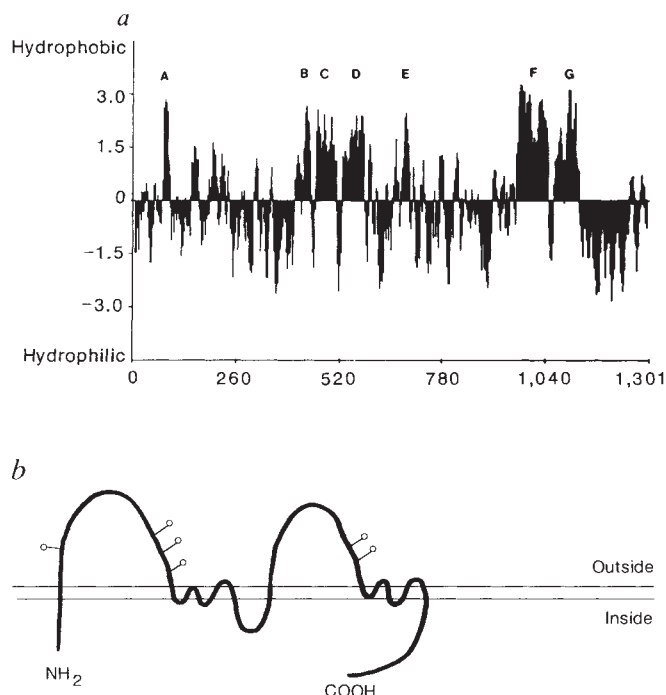


FIG. 6 a, Hydropathy plot of the deduced amino-acid sequence of the Ptc protein using the algorithm of Kyte and Doolittle²⁵ using a window size of 11. The seven hydrophobic regions are designated A–G. b, Schematic two-dimensional representation of a possible model of the Ptc protein in the plasma membrane (not drawn to scale). Predicted N-glycosylation sites are marked with circles. The boundaries of the domains are defined by the charged residues indicated in Fig. 5.

expression that we have described. In stage-10 embryos, all ectodermal cells in each parasegment, except those that express *en*, are found to express *ptc*. Only the latter cells should thus be able to respond to the *wg*-encoded signal by expressing *en*. Of note is that the early repression of *ptc* in the *en*-expressing cells seems to depend on *en* function itself (A.H. and P.W.I., manuscript in preparation), indicating that *ptc* may be a component of a regulatory loop that maintains *en* expression at the parasegmental boundary. Early *en* expression would ensure that cells are competent to respond to a *wg*-encoded signal (by repressing *ptc*), but presumably these cells will respond only if they are in the appropriate location, that is, adjacent to *wg*-expressing cells.

The predicted structure of the Ptc protein indicates several different ways in which it could perform such a function. One possibility is that the *ptc*-expressing cells lose their competence to respond to a *wg*-encoded signal because of an earlier response to some other signal, for which the Ptc protein acts as a receptor. A second possibility is that the expression of *ptc* causes cells to respond differently to the *wg*-encoded signal. For instance, Ptc could change the physiology of expressing cells by mediating a

membrane transport process and hence influence the transduction of the *wg* signal. Alternatively, the Ptc protein could interact directly with the receptor for the *wg* signal, coupling it to a different pathway. It is notable that at the beginning of stage 12, *ptc* expression disappears from cells immediately anterior to the *wg*-expression domain, but persists in two distinct domains in each parasegment. Although the reason for this later regulation is unclear (because *ptc* expression would seem to persist only in those parts of the segment unaffected by its absence), it has important consequences for models that postulate concurrent activity of *wg* and *ptc*, implying that there is a rather narrow period during which cells normally respond to the *wg*-encoded signal.

At this stage we cannot easily discriminate between these various models; further analysis of the structure of the Ptc protein should lead to a better understanding of its function and of the cellular interactions that underlie cell patterning in the larval ectoderm.

Note added in proof: An independent molecular analysis of *ptc* has also been recently described by J. Hooper and M. P. Scott (*Cell*, in the press). □

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Fluvial valleys and martian palaeoclimates

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NETWORKS of small fluvial valleys are extensively developed throughout the ancient heavily cratered terrains of Mars^{1–3}. The existence of the valleys has been cited as compelling evidence for a relatively dense primordial atmosphere capable of maintaining an Earth-like hydrological cycle. Theoretical models of early atmospheric evolution^{4,5} describe the maintenance of a dense CO₂ atmosphere and a warm, wet climate until the end of the heavy-bombardment phase of impacting. However, the presence of very young, Earth-like fluvial valleys on the northern flank of Alba Patera^{6–8} conflicts with this scenario. Whereas the widespread ancient martian valleys generally have morphologies indicative of sapping erosion by the slow outflow of subsurface water^{9,3}, the local Alba valleys were probably formed by surface-runoff processes. Because subsurface water flow might be maintained by hydrothermal energy inputs^{10,11} and because surface-runoff valleys developed late in martian history, when planet-wide climatic conditions were presumably similar to the present, it is not necessary to invoke drastically different planet-wide climatic conditions to explain valley development on Mars. The Alba fluvial valleys can be explained by hydrothermal activity or outflow-channel discharges that locally modified the atmosphere inducing precipitation and local overland flow on low-permeability volcanic ash.

The widespread valley networks of Mars are predominantly associated with the heavy-bombardment phase of cratering history¹², corresponding to the Noachian Period¹³. Local areas of younger valleys are recognized¹⁴, but nearly all martian valleys show remarkably low drainage densities, ranging from 0.015 to 0.16 km per km² (ref. 15). This contrasts with the nearly ubiquitous terrestrial fluvial dissection, in which the minimum observed value is ~1.0 km per km² (ref. 16). The sapping mechanism¹⁷ is generally invoked to explain this and other dissimilarities between martian networks and terrestrial valleys of rainfall-runoff genesis. Sapping occurs when ground water discharges to the surface at a point of geological control (joint, fault or pre-existing valley, for example) and undermines the overburden to generate headward erosion at its point of emergence.

The Noachian martian valleys probably formed before the end of late heavy bombardment (2.8–3.8 Gyr BP) (refs 18–20). The absolute age of the very young valleys on Alba Patera, however, is less constrained (2.6–0.5 Gyr BP) (refs 18, 19). Crater based and geochronological criteria classify Alba as post heavy bombardment¹² and Early Amazonian¹³, respectively. Valleys on Alba Patera were formed both by fluvial processes and by lava-flow processes. Some valleys display multigenetic features indicating that both fluvial and lava-flow processes were important in their formation. Monogenetic fluvial valleys, however, formed only on the northern flank of Alba in regions where lava-flow morphology is less common or absent (Fig. 1). The surface on which these valleys formed is relatively smooth and appears to be mantled, probably by ash^{6,8}. Adjacent to these smooth areas are prominent lava flows, with pristine morphology. Lava tubes and channels formed on these flows, often display a discontinuous, pitted-surface morphology or form distributary patterns^{6,8}. By contrast, fluvial valleys on Alba display a continuous surface morphology and form a complex system of branching tributary networks which are characteristics typical of terrestrial river valleys.

The exact nature of processes causing the local fluvial valleys on the northern flank of Alba is the major concern. A sapping origin has been proposed²¹, but our morphological and terres-

trial analogue studies^{6,8,22} indicate that the characteristics of the fluvial valleys on Alba Patera are more compatible with a formation by surface runoff. Runoff valleys form when surface water flows moving downslope erode into the underlying land surface. Alba valleys have a V-shaped cross-section, and headwater regions that blend in gradually with the surrounding landscape. Some of the larger valleys present in the very high resolution frames (Fig. 2) may show signs of incipient valley enlargement by sapping. Unlike the Noachian valley networks, however, the local Alba valleys do not generally exhibit such sapping attributes as broad flat floors, theatre heads or structural control by faults or joints. Instead, the overall pattern is morphologically similar to that of valleys formed by rainfall runoff on Hawaiian volcanoes (Fig. 1).

We propose that the Alba valleys developed in a manner similar to fluvial valleys formed on terrestrial volcanoes. On Earth, fluvial valley development on volcanic landscapes is a progressive sequence of formation by surface runoff and subsequent modification by sapping. The rate at which these processes proceed is a function of climate, time, lithological properties, slope and stream process (surface runoff or ground-water sapping)^{23,22}. In general, fluvial valleys cannot form on volcanic landscapes until the permeability of the basalt flows is reduced either through the emplacement of an aeolian mantle, ash mantle or by weathering of the basalt surface^{6,8,22}. When the rate of infiltration becomes less than the rate of runoff, valleys are formed by surface runoff. On Alba Patera, fluvial runoff valleys could have formed only in regions where surface water supply and lithological conditions were amenable to runoff. These factors resulted in drainage densities for local Alba valleys of 0.3–1.5 km per km², an order of magnitude higher than generally observed for the Noachian valleys. These values are comparable to measurements on the dry leeward side of Hawaiian volcanoes, including Kilauea (0.6–3.0 km per km²), Mauna Kea (0.3–5.0 km

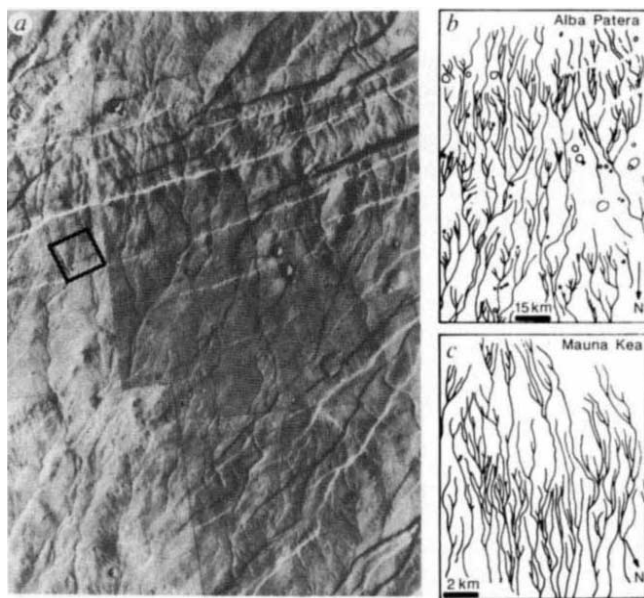


FIG. 1 *a*, High-resolution photomosaic of fine-textured fluvial valleys on the northern flank of Alba Patera. Valleys are formed in a region where lava-flow morphology is absent, indicating that the region was mantled, probably by ash. These valleys are the most highly integrated fluvial valleys on the surface of Mars. The high degree of fluvial (runoff) valley development on such a young volcano indicates that a mechanism capable of providing sustained surface water flow (at least locally on Alba) operated long after the end of the heavy-bombardment phase of impacting. Downslope direction is toward the north (from Viking photomosaic: MTM 45107) (Boxed area denotes location of image in Fig. 2). *b*, Drainage map of fluvial valleys in *a*. The Alba valleys form highly integrated transitional parallel-to-dendritic drainage patterns similar to those formed by rainfall runoff on the Hawaiian volcanoes (such as *c*, Mauna Kea).

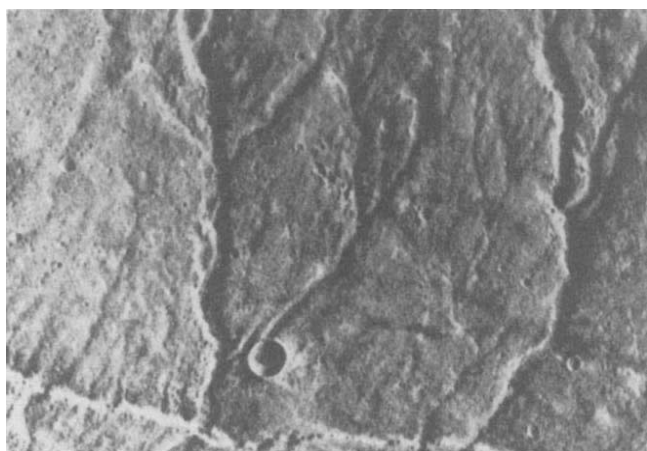


FIG. 2 Very-high-resolution images (7 m per pixel) (see location in Fig. 1a) of the fluvial valleys on Alba Patera. The tapered valley heads (see also Fig. 1a) which blend in gradually with the surrounding terrain, V-shaped cross-valley profiles (when viewed in stereo) and the lack of structurally controlled drainage patterns suggest a formation by surface runoff. When viewed in stereo, the larger valleys in this photograph seem much deeper than the others and have downcut into resistant subsurface layers (that is, basalt or welded ash). If these layers are permeable basalt, then some of this enlargement may reflect incipient sapping processes. However, the cross-valley profiles are distinctly V-shaped indicating that surface runoff was still the dominant process when valley formation ceased. (Diameter of large crater is ~ 1 km) (Viking frames: 445B07–08).

per km^2) and Kohala (0.2–2.8 km per km^2) (refs 7, 22).

In Hawaii, valleys evolve with time as the low-permeability ash and weathered lava are eroded. Deep incisions tap ground water and the valleys transform to a sapping morphology^{24,3,6,22}. On Mars, fluvial valleys are also present on the volcanoes Ceraunius Tholus and Hecates Tholus, which formed before the end of late heavy bombardment^{12,18,19} (Fig. 3). Drainage-density values for valleys on all three volcanoes are similar, but the Alba valleys are better integrated. Valleys on Ceraunius and Hecates have initially formed by surface runoff but, unlike the Alba valleys, the Ceraunius and Hecates valleys were extensively enlarged or reactivated, probably by sapping. The less integrated (and therefore less developed) nature of the fluvial valleys on Ceraunius and Hecates suggests that less surface water was available during valley development. The presence of extensive valley modification on these volcanoes indicates, however, that fluvial valleys developed over a very long time. Long periods ($> 10^5$ yr) of ground-water flow are needed to produce the degree of valley modification present. Such sustained flow would have required a recycling mechanism.

The indicated history of martian fluvial-valley formation poses an interesting dilemma. The widespread Noachian valleys are less indicative of atmospheric hydrological cycling than are the local Alba valleys of anomalously young age. Because water can flow as ice-covered rivers on Mars even for present-day atmospheric conditions²⁵, it is the sustained discharge to the surface that is the limiting factor in valley formation on Mars. For sapping valleys, flow can be maintained either by recharge from atmospheric precipitation or by circulation in subsurface hydrothermal systems. Atmospheric-water transfer may only be required when valley morphology indicates surface-water runoff processes.

A variety of mechanisms seem capable of supplying precipitable water to the local vicinity of Alba under mean climatic conditions not drastically different from those of today. The ultimate water source is the extensive subsurface reservoir indicated by abundant geomorphological evidence of ground water and ice^{26,3}. Vapour from eruptions and explosive activity emanating from Alba Patera could have transported some of

this water into the surface environment²¹. Even more important for valley formation would be the development of prolonged hydrothermal circulation within the water or ice-rich subsurface materials underlying the low-relief volcano. Hydrothermal systems are capable of melting ground ice, focusing the resulting flow of ground water into narrow effluent zones and then transporting these fluids to the surface (by seeps and springs for example). Ground water can be recirculated through these systems for several million years¹¹. By comparison, fluvial valleys on the Hawaiian volcanoes generally formed in $\sim 10^4$ – 10^5 yr (refs 8, 22). Therefore such circulation should have persisted over the timescales needed for fluvial valley formation on Alba Patera. Also, as on Earth, locally warmer, more moist environments might have developed near regions of hydrothermal activity and thereby induced areas of enhanced precipitation along the perimeter of these zones. Even snow accumulation in such areas could have yielded surface water flow under present climatic conditions²⁷.

Because the outflow channels developed at approximately the same time as Alba¹², an additional source of atmospheric moisture may have been derived from outflow discharges²⁸. Using the present topography²⁹, the minimum amount of water required to erode the outflow channels²⁶ could have formed a temporary circumpolar body of water on the northern martian plains with an average depth of 660 m and a surface area of $1.1 \times 10^7 \text{ km}^2$. This assumes that water lost to evaporation and infiltration was equivalent to the volume of water in excess of the minimum amount of water required to erode the outflow channels. The climatic influence of the hypothesized circumpolar sea and its ice cover would have been a temporary aberration from mean, planet-wide conditions. The prevailing northerly winds at this latitude³⁰ would move saturated air from this sea to Alba, where orographic precipitation might occur as

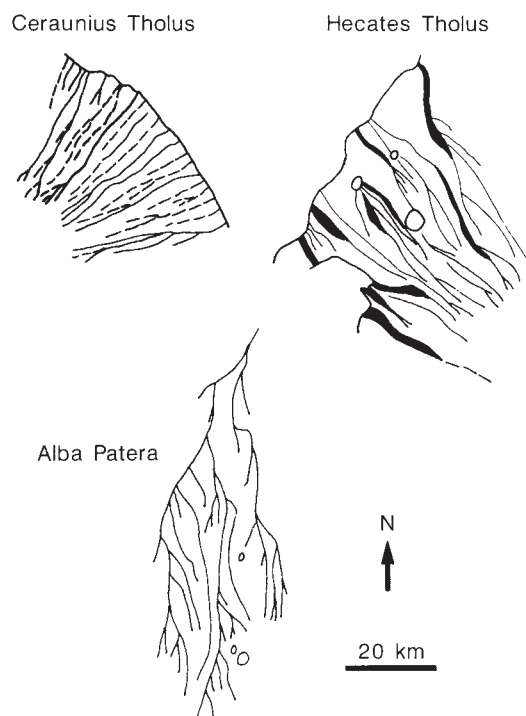


FIG. 3 Comparison figure of representative drainage patterns on the martian volcanoes: Ceraunius Tholus, Hecates Tholus and Alba Patera. Well developed drainage patterns on Alba indicate that considerable water was present at the surface during fluvial valley formation. The lack of reactivation (as is apparent on Ceraunius (solid lines)) or enlargement (as is apparent on Hecates (shaded black areas)) of the valleys on Alba, however, suggests that the period of fluvial valley activity on Alba was relatively short-lived.

on the windward flanks of the Hawaiian volcanoes. Whatever the precipitation mechanism, water would rapidly infiltrate the highly permeable volcanic rocks, precluding surface flow except in the area where low permeability ash mantles the northern flanks of Alba. There, the local occurrence of surface runoff would lead to the distinctive Earth-like valley pattern (Fig. 1).

The anomalously young age and the surface-runoff origin of the Alba valleys have important implications for understanding the climate history of Mars. Whatever the local mechanisms of

their origin, the Alba valleys formed late in martian history when mean planet-wide conditions were presumably similar to those at present. It seems inappropriate therefore, to assume for models of martian atmospheric evolution^{4,5} that a persistent mean climatic epoch drastically different from that of today is required to explain the valley networks that formed during the heavy bombardment period. On Mars, as on Earth, valley genesis may occur through a variety of local processes. □

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Latitudinal variations in plankton $\delta^{13}\text{C}$: implications for CO_2 and productivity in past oceans

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THE stable-carbon isotopic composition of marine organic material has varied significantly over geological time, and reflects significant excursions in the isotopic fractionation associated with the uptake of carbon by marine biota^{1–8}. For example, low $^{13}\text{C}/^{12}\text{C}$ in Cretaceous sediments has been attributed to elevated atmospheric (and hence oceanic) CO_2 partial pressures^{3,4,8}. A similar depletion in ^{13}C in present-day Antarctic plankton^{2,9–12} has also been ascribed to high CO_2 availability^{3,4}. We report, however, that this high-latitude isotope depletion develops at CO_2 partial pressures ($p\text{CO}_2$ levels) that are often below that of the present atmosphere (340 μatm), and usually below that of equatorial upwelling systems (> 340 μatm). Nevertheless, because of the much lower water temperatures and, hence, greater CO_2 solubility at high latitude, the preceding $p\text{CO}_2$ measurements translate into Antarctic surface-water CO_2 (aq) concentrations that are as much as 2.5-times higher than in equatorial waters. We calculate that an oceanic $p\text{CO}_2$ level of > 800 μatm (over twice the present atmospheric $p\text{CO}_2$) in a warmer low-latitude Cretaceous ocean would have been required to produce the plankton ^{13}C depletion preserved in Cretaceous sediments.

Plankton of the South Atlantic Ocean and Weddell Sea (Antarctica) exhibit a dramatic decrease in $\delta^{13}\text{C}$ as a function of both latitude and sea surface temperature (Fig. 1b). This isotope trend does not parallel the relatively constant $\delta^{13}\text{C}$ of the associated total dissolved inorganic carbon TCO_2 (Fig. 1a). Because some small fraction of this TCO_2 provides a substrate for plankton biomass formation (through phytoplankton carbon

fixation), the increasing difference in isotopic composition between plankton and TCO_2 therefore requires a fundamental change in the carbon biogeochemistry and associated isotope fractionation with changing temperature and latitude.

The strong relationship between temperature and plankton $\delta^{13}\text{C}$ was initially interpreted as a direct temperature effect on plankton isotope abundances, possibly resulting from temperature-sensitive changes in the kinetic isotope effect associated with phytoplankton CO_2 fixation^{9,13,14}. However, subsequent laboratory experimentation^{15–18} as well as further field sampling and analysis^{13,14,19} failed to find consistent trends with either controlled temperature or ocean temperature. Large interspecies differences in phytoplankton $\delta^{13}\text{C}$ response to temperature were noted^{15,18}, and species and water-mass effects were later used to interpret some of the isotope variability observed in the ocean^{2,13,14,19,20}. The significant ^{13}C depletion in Southern Ocean plankton, however, remained largely unexplained.

Other experiments demonstrated a strong relationship between external CO_2 concentration and $\delta^{13}\text{C}$ in marine plants^{15–17,21}. It was shown that increasing CO_2 availability generally results in a decrease in plant $\delta^{13}\text{C}$ and an increase in the difference between the $\delta^{13}\text{C}$ of the plant and that of the CO_2 it uses. This difference, $\Delta_{\text{c-p}} (= \delta^{13}\text{C}_{\text{CO}_2} - \delta^{13}\text{C}_{\text{plant}})$, expresses the overall fractionation factor associated with carbon fixation by plants. $\Delta_{\text{c-p}}$ has been observed to range from near 0‰ at low CO_2 concentrations to as high as 28‰ at CO_2 concentrations of > 5% CO_2 (v/v in air) (refs 21, 22). This has led to speculation that high $p\text{CO}_2$ was responsible for the low $\delta^{13}\text{C}$ and higher apparent $\Delta_{\text{c-p}}$ values indicated in the marine sedimentary record^{1,3,4,8,17,21} and observed in contemporary Antarctic plankton^{3,4}.

Figure 1c shows, however, that $p\text{CO}_2$ levels in Antarctic surface waters are not significantly elevated relative to those at lower latitudes. In fact $p\text{CO}_2$ levels in the Weddell Sea are frequently much lower than those encountered in equatorial waters. We point out, however, that to relate $p\text{CO}_2$ to the dissolved CO_2 concentration, $[\text{CO}_2(\text{aq})]$, temperature-sensitive CO_2 solubility as described by Henry's law must be considered as follows:

$$[\text{CO}_2(\text{aq})] = a \times p\text{CO}_2 \quad (1)$$

Using known solubility constants, α (ref. 23), the calculated ranges of $[\text{CO}_2(\text{aq})]$ for the South Atlantic and Weddell Sea show, on average, a dramatic increase in dissolved CO_2 concentration with decreasing temperature and increasing latitude (Fig. 1b). Again, these increases are due to the greater solubility of CO_2 at lower water temperatures for a given $p\text{CO}_2$ level, a relationship that is closely approximated by a second-order polynomial equation (Fig. 1b).

The above observation of as much as 2.5 times more $[\text{CO}_2(\text{aq})]$ at high latitudes relative to that of low latitudes may then be causing the plankton $\delta^{13}\text{C}$ trends with latitude and temperature. This could occur because $\Delta_{\text{c-p}}$ increases with increasing $[\text{CO}_2(\text{aq})]$ ^{15-17,21,22,24,25} or because $\Delta_{\text{c-p}}$ remains constant but the $\delta^{13}\text{C}$ of both the CO_2 and the phytoplankton increase as a significant fraction of the CO_2 is converted to biomass following the Rayleigh distillation model of a closed system^{26,27}. An increase of $\Delta_{\text{c-p}}$ with increasing $[\text{CO}_2(\text{aq})]$ (the first mechanism) may involve a corresponding decrease in the relative importance of bicarbonate (rather than CO_2) as an initial substrate for phytoplankton carbon fixation^{20,22,24}. It could also involve reduced phytoplankton demand for CO_2 at high latitude. Estimates of open-water primary production (net CO_2 uptake) in the Weddell Sea of $10\text{--}20\text{ g C m}^{-2}\text{ yr}^{-1}$ (refs 28, 29) are significantly lower than those of equatorial Atlantic waters. A reduced internal demand for CO_2 in combination with elevated external CO_2 concentration may then result in greater expression of the kinetic isotope fractionation associated with CO_2 fixation²² and therefore lower plankton $\delta^{13}\text{C}$ at high latitude.

If changes in phytoplankton species composition and associated $\Delta_{\text{c-p}}$ values^{14,18,20} were contributing to the observed variation of $\delta^{13}\text{C}$ with latitude, one would expect to see non-uniformities or breaks in the plankton $\delta^{13}\text{C}$ trend with temperature and latitude as various water-mass regimes and phytoplankton species compositions were transected in the Southern Ocean. Such breaks are not evident in Fig. 1b, nor are they apparent in the plankton isotope-temperature ($<25^\circ\text{C}$) trend reported in ref. 14.

A linear relationship between marine plankton $\delta^{13}\text{C}$ and $[\text{CO}_2(\text{aq})]$ is indicated by their similar trend with temperature (Fig. 1b), as has been previously suggested³⁰. Changes in freshwater plankton $\Delta_{\text{c-p}}$, however, have been described as a linear function of $\log [\text{CO}_2(\text{aq})]$ based on extensive experimental and field measurements in several New Zealand lakes²⁵. However, determination of the true relationship between *in situ* marine

plankton $\delta^{13}\text{C}$ and $[\text{CO}_2(\text{aq})]$ (and therefore possible insight into the biogeochemical mechanism(s) involved) will require simultaneous measurement of these two variables over broad temporal and spatial scales in the ocean, data which are presently unavailable.

In the meantime, we suggest that much of the carbon isotope variability of plankton observed in the Southern Ocean, if not elsewhere, is directly related to variations in $[\text{CO}_2(\text{aq})]$. A strong relationship between temperature and plankton $\delta^{13}\text{C}$, sometimes evident in past field (for example, Fig. 1b) and laboratory studies, may have been an indirect one, resulting from the aforementioned temperature effects on CO_2 solubility and concentration. It is not easy to address this point using the data of many of these past studies because $p\text{CO}_2$, % CO_2 in feed gas and/or TCO_2 concentrations were reported rather than the more relevant $[\text{CO}_2(\text{aq})]$.

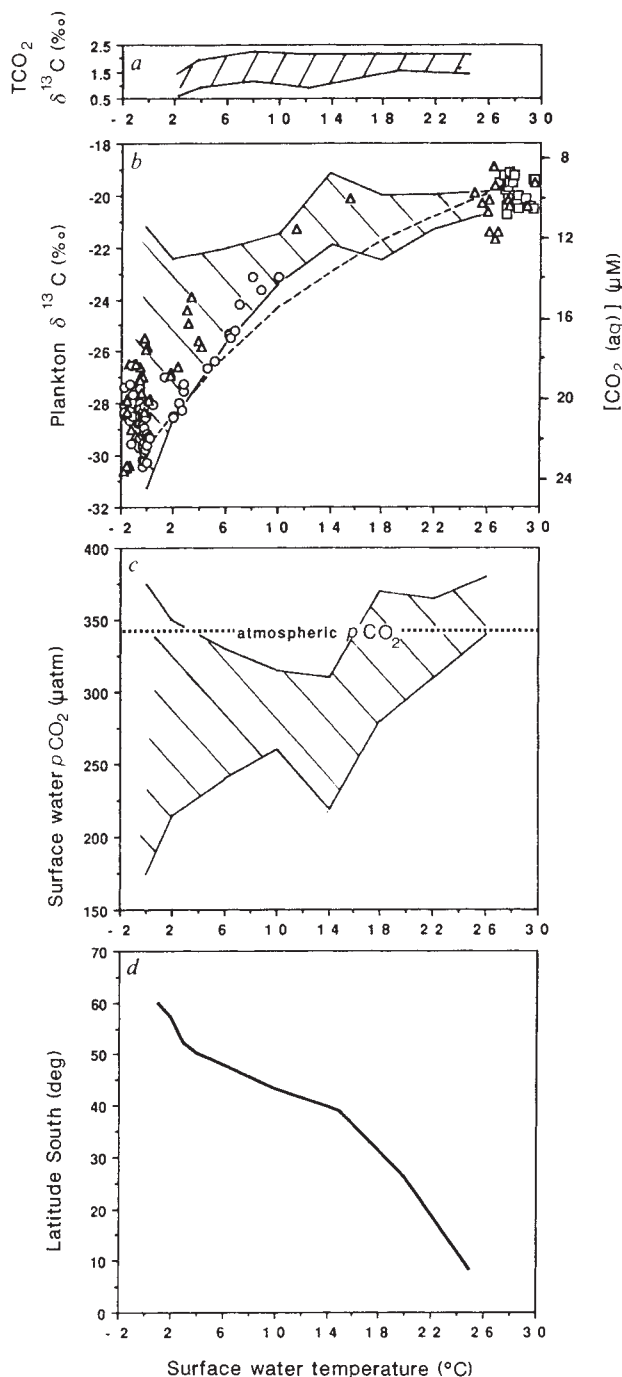


FIG. 1 a, Range of total dissolved inorganic carbon (TCO_2) $\delta^{13}\text{C}$ as a function of surface water temperature in the South Atlantic and Southern Oceans³⁵. $\delta^{13}\text{C} = \{R_{\text{sample}}/R_{\text{PDB std}} - 1\} \times 1,000(\text{‰})$ where $R = {}^{13}\text{C}/{}^{12}\text{C}$ of the sample or the PDB carbonate standard. The analytical precision of these measurements is typically $\pm 0.2\text{‰}$. b, Plankton $\delta^{13}\text{C}$ and $[\text{CO}_2(\text{aq})]$ as a function of surface water temperature in the S. Atlantic and Southern Oceans, and in the Weddell Sea. Isotope and temperature data from refs 2 and 9 (triangles), ref. 14 (squares), and G.H.R., unpublished data (circles). Shaded-area is the range of $[\text{CO}_2(\text{aq})]$ as a function of temperature as calculated from the $p\text{CO}_2$ data (Fig. 1c) using Henry's law (equation (1)) and known solubility constants²³ assuming a salinity of 34 p.p.t. The dashed line represents surface-water equilibrium values under an atmospheric $p\text{CO}_2$ of 340 μatm. As a function of sea surface temperature T , this dashed line is closely described by the equation $[\text{CO}_2(\text{aq})]_{\text{equil}} = 22.04 - 0.77T + 0.01T^2$. Note that $[\text{CO}_2(\text{aq})]$ is graphed to increase from top to bottom to emphasize its apparent inverse relationship with plankton $\delta^{13}\text{C}$. Based on their common trend with temperature, the relationship between plankton $\delta^{13}\text{C}$ and $[\text{CO}_2(\text{aq})]$ seems to be first order and may be approximated by the equation: Plankton $\delta^{13}\text{C} = -0.8[\text{CO}_2(\text{aq})] - 12.6$ as determined by the relationship between the scales of the left and right vertical axes. c, Range of surface sea water $p\text{CO}_2$ at ambient temperature as a function of sea water temperature in the S. Atlantic, Southern Ocean and Weddell Sea³⁶. A modern atmospheric $p\text{CO}_2$ of 340 μatm is indicated. d, Approximate latitude corresponding to a given mean sea surface temperature in the S. Atlantic and Southern Oceans.

Present-day Antarctic plankton and the sedimentary remains of Cretaceous-age marine plankton communities have several features in common. As well as their similar low $\delta^{13}\text{C}$ values^{3,4}, reoccurring organic-rich horizons within Cretaceous marine sediments and Antarctic plankton have unusually low $^{15}\text{N}/^{14}\text{N}$ ratios (refs 11, 12, 31), and very low abundances (or little evidence) of carbonate-forming plankton⁴. Furthermore, low phytoplankton productivity probably characterized the Cretaceous Atlantic Ocean³² as it does present-day Antarctic waters^{28,29}. Although atmospheric and ocean $p\text{CO}_2$ levels were probably elevated in the Cretaceous^{33,34}, only low-temperature, high-latitude marine systems today provide $\text{CO}_2(\text{aq})$ concentrations and therefore plankton $\delta^{13}\text{C}$ values similar to those that were apparently present in much of the warmer^{33,34} Cretaceous ocean. A Cretaceous sediment mean $\delta^{13}\text{C}$ value of -27% (refs 3, 4) would, as a plankton $\delta^{13}\text{C}$ value in today's ocean, correspond to a $[\text{CO}_2(\text{aq})]$ of $\sim 20\ \mu\text{M}$ (Fig. 1b). Using equation (1), the oceanic $p\text{CO}_2$ required to generate a similar $[\text{CO}_2(\text{aq})]$ in a warmer (32°C , for example), low-latitude Cretaceous ocean would be $>800\ \mu\text{atm}$ or more than twice the $p\text{CO}_2$ of the current atmosphere. This is within the range of the 2–13 times higher $p\text{CO}_2$ previously estimated^{33,34} to exist globally during the Cretaceous period. Further research on the relationship between plankton $\delta^{13}\text{C}$ and $\text{CO}_2(\text{aq})$ concentration may lead to refinements in our understanding of CO_2 biogeochemistry in both past and present oceans. □

Direct dating of Phanerozoic sediments by the ^{238}U – ^{206}Pb method

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DIRECT radiometric age determination of Phanerozoic carbonates has been a long-standing problem in geochronology. Rb–Sr and K–Ar dating schemes, commonly used to constrain the chronology of Phanerozoic sediments, have so far proved to be unsuccessful in dating these rocks because of their poor enrichment in radiogenic ^{87}Sr and ^{40}Ar and also because of analytical difficulties. Recent studies^{1,2} have demonstrated that large amounts of radiogenic Pb exist in some carbonates and that they could be dated by the Pb–Pb method. However, the precision of this method is severely limited to samples having very high uranium to lead ratios because by the beginning of the Phanerozoic (~ 590 Myr ago) 98% of ^{235}U originally present in the Earth had decayed to ^{207}Pb . Here we report the first isochron age of carbonates using the ^{238}U – ^{206}Pb method on corals. This method, which gives good accuracy even for low U concentrations, has great potential for the direct dating of sedimentary sequences and should be valuable for refining the Phanerozoic timescale.

We obtained samples from the late Middle Devonian (Givetian) sediments of the Thedford–Arkona region in south-western Ontario, Canada. The area consists of a 27-m section of fossiliferous shale and limestone of the Hamilton group which has been subdivided into three units: the Arkona, Hungry Hollow and Widder formations. Lithological and biostratigraphic correlation of the rocks is straightforward and is summarized in the stratigraphic section in Fig. 1 (refs 3 and 4). The Arkona formation consists of 12 m of a soft bluish calcareous shale. Two parts of the Hungry Hollow formation may be distinguished—0.5 m of encrinal limestone overlain by a 'coral zone', and a 1-m bed of coral-rich shale. The Widder formation consists of 13 m of shales interbedded with highly fossiliferous limestone. The sediments of this area are well preserved and the burial temperature was probably no more than 150°C (ref. 5).

We sampled rugose corals of the genera *Heliophyllum* and *Cystiphyllodes* from the 'coral zone' of the Hungry Hollow member. Their remains consist of secondary low-magnesian calcite in the form of replaced skeletal elements, infilling micrite and subordinate sparry calcite. Very small amounts of authigenic pyrite are disseminated throughout the corals. We also sampled the limestone from the lower part of the Hungry Hollow formation (Ark-14), limestone from the overlying Widder formation (Ark-4) and pyrite infilling from a brachiopod (*Mucrospirifer*) in the Arkona shale (Ark-3).

We measured Pb, U and Th using a custom-built 30-cm 90° -sector mass spectrometer. Details on the chemical extraction techniques will be presented elsewhere. We determined concentrations of U, Th and Pb by isotope dilution using a mixed ^{235}U – ^{230}Th – ^{208}Pb tracer (see Table 1). The Pb and U blanks were 190–410 and 12–20 pg, respectively. We calculated the ages using the decay constants recommended in ref. 6 and obtained the isochrons using the line-fitting method in ref. 7. The uncertainty limits drawn on the diagrams are at the 2σ level.

Bulk Pb concentrations for the corals range from 200–300 p.p.b. (parts per 10^9), levels very similar to modern corals⁸. Rugose corals became extinct in the Permian so we cannot make direct comparisons with extant unaltered taxa. These corals differ from most modern Scleractinian corals in that they made their skeletons of magnesian carbonate rather than aragonite⁹. Compared to sea water, however, modern corals of both types of CaCO_3 are known to concentrate Pb up to 300 times more

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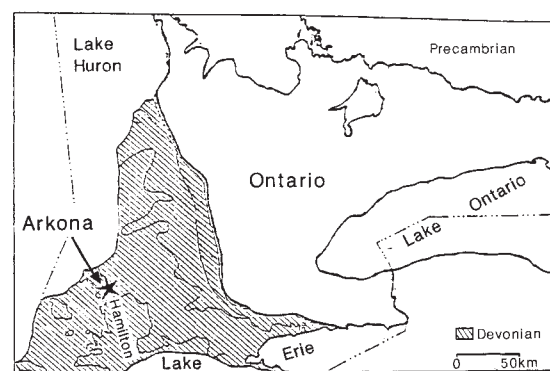
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than Ca (ref. 10). Thus, the Pb concentration levels in the rugose corals could reflect original Pb precipitated from Devonian sea water without diagenetic modification. Bulk U contents in the rugose corals range from 46–82 p.p.b., much lower than the levels of 1–3 p.p.m. found in modern aragonitic corals¹¹. Little is known about the U contents of modern magnesian calcite coral skeletons. It is well known that assimilation of U by marine organisms and its resultant abundance in lithified sediments involves both biological and diagenetic controls^{10,12}. The low U contents measured in rugose corals may be attributed in part to loss during diagenetic transformation of their skeletons to low-magnesian calcite. Without knowledge of the original concentrations, however, the degree to which this has happened is uncertain. In any case, the μ ($^{238}\text{U}/^{204}\text{Pb}$) values of rugose corals are much lower than those of modern corals.

The isotope data for the corals form a linear trend on the Pb isotope correlation plot (Fig. 2a) but there is insufficient radiogenic Pb (maximum 2.7%) to define a precise Pb isochron. On a plot of $^{206}\text{Pb}/^{204}\text{Pb}$ against $^{238}\text{U}/^{204}\text{Pb}$ (Fig. 3), however, data for the corals form a nine-point isochron corresponding to an age of 376 ± 5 Myr (1σ). The uncertainty has been adjusted to include the scatter which slightly exceeds analytical limits ($S/(n-2) = 4.7$ where S is the sum of the squares of the deviation and n the number of points). The data point for the Arkona-shale pyrite from the brachiopod lies significantly below the isochron, indicating a different age and/or source for this Pb. By contrast, authigenic pyrite from a coral (Ark-10s) falls on the least radiogenic end of the isochron indicating that this sulphide is cogenetic with the calcite phases and the other corals. We infer from this relationship that adequate ranges in μ values exist and a precise age may be obtained from a single specimen of coral. The value for sample Ark-8 also falls below the isochron. This sample has an anomalously high value of Pb (1.2 p.p.m.) and is correlated with a relatively large amount of visible pyrite in this coral. The simplest explanation for this point is that there has been an addition of Pb to the coral in the form of pyrite of a composition similar to that of the brachiopod.

An isochron for the same points using the ^{235}U – ^{207}Pb decay scheme (data not shown) gives a less precise age of 427 ± 57 Myr (1σ). Despite the poor precision which is a result of the lack of enrichment in radiogenic ^{207}Pb , the age is concordant with the ^{238}U – ^{206}Pb age. Thus, as the isochrons are preserved and both ages agree, the U–Pb system in the corals has remained closed with respect to parent and daughter isotopes.



The Devonian of Southern Ontario

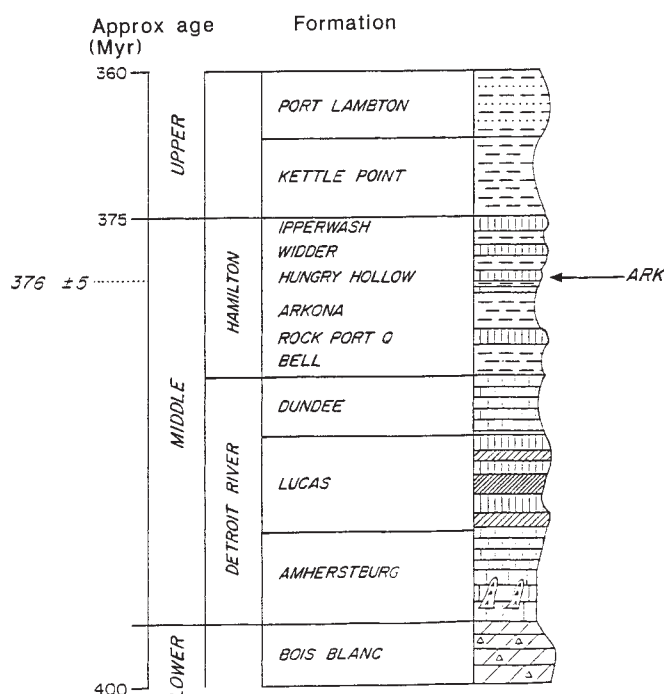


FIG. 1 Stratigraphic section of the Devonian rocks of Southern Ontario³. Timescale is from ref. 14.

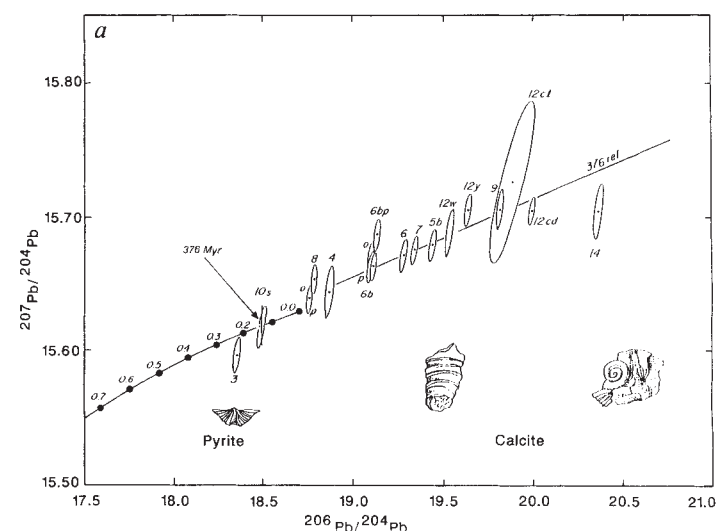
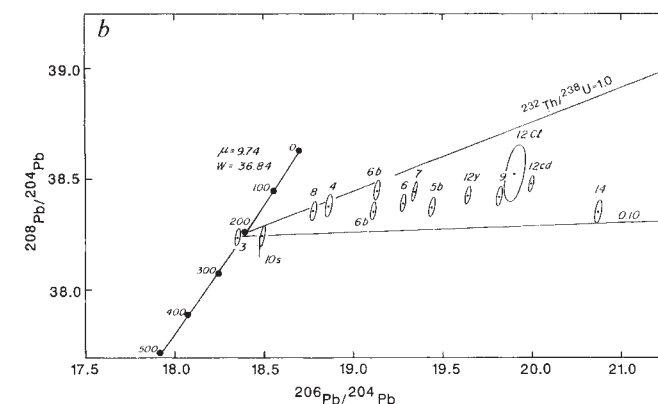


FIG. 2 a, Pb–Pb isotope correlation plot for corals, rocks and pyrite from brachiopod from formations of the Arkona area. Uncertainty ellipses are given at the 95% confidence level. Initial Pb isotopic ratios for the corals



are indicated with a cross. Reference curve from ref. 16. b, $^{208}\text{Pb}/^{204}\text{Pb}$ – $^{206}\text{Pb}/^{204}\text{Pb}$ correlation plot for Arkona corals. Straight lines show $^{232}\text{Th}/^{238}\text{U}$ values of 1.0 and 0.1. Reference curve is from ref. 16.

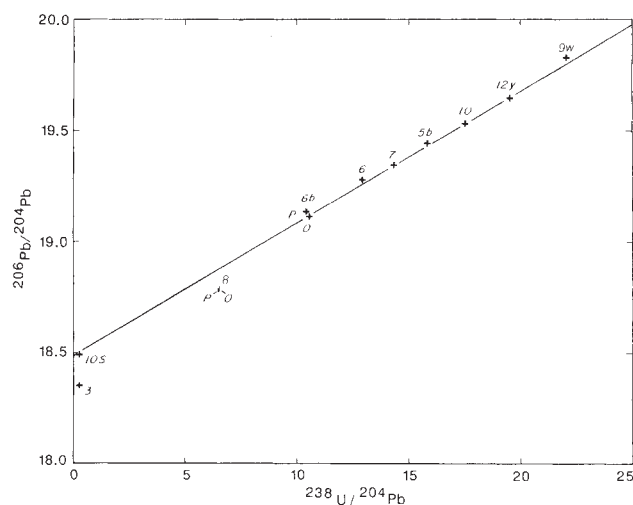


FIG. 3 ^{206}Pb - ^{238}U isochron plot for the corals giving an age of 376 ± 5 Myr. Ark-8 (shown in duplicate) was not used in the regression of the points. Ark-3 represents the pyrite analysis from the brachiopod from the Arkona shale.

By contrast to the *Heliophyllum* corals, the specimen of *Cystiphyllodes* shows some age discordance. The data point for the bulk sample (Ark-12w) lies well above the ^{238}U - ^{206}Pb isochron (data not shown). This coral has a much more porous structure and has numerous air pockets that are lined with large amounts of sparry calcite. This calcite (Ark-12sp) has very low U and Pb concentrations—6.8 and 21.3 p.p.b., respectively. The data point for this sample as well as that for micrite (Ark-12cd) also lie above the isochron. However, data for yellow ferroan calcite (Ark-12y), peculiar to this specimen, lies on the isochron. The U-Pb system of the latter calcite is closed, possibly because it was not in contact with any air pockets. The spaces may have facilitated movement of fluids concomitant with calcite recrystallization long after burial and initial closure of the U-Pb system.

ization long after burial and initial closure of the U-Pb system.

The U and Pb contents from limestones from enclosing strata are 0.35–0.80 and 1.6–3.1 p.p.m., respectively. These values are much higher than the averages of these elements in the corals, and are within the range of limited data from other Phanerozoic limestones^{2,13}. The isotope data for the limestones from Arkona indicate that they had slightly lower initial ratios of $^{208}\text{Pb}/^{204}\text{Pb}$, $^{207}\text{Pb}/^{204}\text{Pb}$ and $^{206}\text{Pb}/^{204}\text{Pb}$.

Figure 2b shows a plot of $^{208}\text{Pb}/^{204}\text{Pb}$ against $^{206}\text{Pb}/^{204}\text{Pb}$. The data for the corals form a trend having a very shallow slope indicating that they have very low $^{232}\text{Th}/^{238}\text{U}$ ratios. This is a reflection of the low enrichment of Th in the rocks and implies that there is insufficient enrichment in ^{208}Pb to date the corals using the ^{232}Th - ^{208}Pb system.

The isochron age of 376 ± 5 Myr compares very favourably with the boundaries which range from 385 to 375 Myr inferred for Givetian strata¹⁴. An idea of the relative timescale for the diagenetic alteration of the corals is provided by a trace element and stable isotope study¹⁵. Based on the similarity of ^{18}O values in texturally well preserved (primary) brachiopods and texturally altered corals it was concluded that contemporaneous sea water was the predominant component of the diagenetic fluids affecting these sediments. Thus, it seems that diagenesis occurred and the U-Pb systems in the corals became closed soon after deposition of the host sediments.

The initial Pb ratios derived from the isochron plots allow a characterization of the source of Pb in these corals. Figure 2 shows that both initial $^{206}\text{Pb}/^{204}\text{Pb}$ and $^{208}\text{Pb}/^{204}\text{Pb}$ ratios are relatively radiogenic compared to those ratios predicted by an average Earth growth curve at 376 Myr (ref. 16). These isotope patterns are similar to marine pelagic sediments¹⁷ and both signatures reflect the high U/Pb and Th/Pb ratios of the upper continental crust.

Closed-system U-Pb behaviour in whole rocks and minerals is comparatively rare. Discordance is usually attributed to the mobility of U, and less frequently Pb, under surface and sub-surface conditions^{18,19}. Compared to the material tested in those studies the corals have concentrations of U and Pb that are

TABLE 1 U, Th and Pb abundances and isotopic compositions of Devonian fossils from Arkona

Sample number	Type	Concentration (p.p.b.)				Atomic ratios					
		U	Th	Pb	^{204}Pb	$^{238}\text{U}/^{204}\text{Pb}$	$^{232}\text{Th}/^{204}\text{Pb}$	$^{232}\text{Th}/^{238}\text{U}$	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$
Ark-3py	Mucr	227	134	60,190	810	0.2385 (7)	0.146 (14)	0.61 (6)	18.355 (5)	15.597 (7)	38.244 (20)
Ark-4	R	350	—	3,086	41.1	7.249 (27)	—	—	18.870 (9)	15.645 (10)	38.387 (25)
Ark-5wb	Helio	78.4	—	315	4.17	15.991 (38)	—	—	19.446 (5)	15.681 (6)	38.382 (24)
Ark-6w	Helio	57.0	—	282	3.74	12.973 (33)	—	—	19.284 (5)	15.672 (6)	38.399 (20)
Ark-6wob	Helio	82.4	—	501	6.65	10.537 (21)	—	—	19.117 (5)	15.664 (6)	38.365 (19)
Ark-6wob*†									19.097 (5)	15.669 (6)	—
Ark-6wpb*		82.0	—	502	6.66	10.482 (18)	—	—	19.136 (7)	15.688 (7)	38.455 (22)
Ark-6wpb*†									19.095 (7)	15.661 (6)	—
Ark-7w	Helio	63.8	—	287	3.79	14.307 (33)	—	—	19.344 (7)	15.677 (6)	38.450 (21)
Ark-8w	Helio	119	—	1,169	15.6	6.508 (15)	—	—	18.785 (6)	15.654 (6)	38.360 (21)
Ark-8wp*†		120	—	1,178	15.7	6.474 (15)	—	—	18.761 (5)	15.640 (6)	—
Ark-9w	Helio	74.7	—	219	2.88	22.103 (49)	—	—	19.823 (6)	15.706 (10)	38.430 (21)
Ark-10w	Helio	81.7	—	300	3.96	17.558 (38)	—	—	19.525 (5)	15.688 (6)	38.426 (44)
Ark-10s	Helio	290‡	—	65,000‡	880	0.2789 (9)	2.38 (46)	8.5 (20)	18.493 (8)	15.619 (20)	38.251 (24)
Ark-12w†	Cyst	46.2	—	242	3.20	12.270 (27)	—	—	19.529 (8)	15.684 (9)	—
Ark-12cd	Cyst	57.4	—	202	2.65	18.459 (42)	—	—	20.004 (9)	15.705 (6)	38.483 (20)
Ark-12sp	Cyst	6.8	—	21.3	0.283	20.34 (23)	—	—	19.907 (33)	15.728 (31)	38.529 (66)
Ark-12y	Cyst	31.6	—	104	1.38	19.559 (48)	—	—	19.647 (6)	15.707 (6)	38.432 (21)
Ark-14	R	797	—	1,595	20.9	32.468 (85)	—	—	20.373 (7)	15.705 (11)	38.357 (27)

Type, Mucl, *Mucrospirifer* sp.; Helio, *Heliophyllum* sp.; Cyst, *Cystiphyllodes* sp.; py, pyrite; R, rock (HCl soluble); b, analysis from same specimen; w, bulk sample; cd, micrite; sp, spar; y, yellow carbonate; 1σ uncertainties in isotope ratios (in parenthesis) account for errors associated with mass fractionation, blank and tracer solutions and refer to the last digit(s) after the decimal.

* Duplicate analysis from solution aliquot.

† Ratios determined from spiked aliquot.

‡ Concentrations are approximations.

orders of magnitude lower. Perhaps at these low levels lattice-bound U and Pb are quantitatively retained in minerals. $\square$

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Hydrogen-isotope evidence for extrusion mechanisms of the Mount St Helens lava dome

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FROM October 1980 to October 1986 the dacite lava dome at Mount St Helens grew through the accumulation of more than 15 separate lobes during extrusive episodes that lasted from 1 to 24 days, except for an anomalous period of continuous growth throughout most of 1983¹. Over these six years, the growth rate was generally volume predictable; that is, the amount of lava added to the dome in a given eruption was roughly proportional to the time since the preceding extrusion². These well documented eruptions were separated by repose intervals lasting from several months to more than a year². In an attempt to identify the triggering mechanisms for these periodic eruptions, we have used hydrogen isotope analyses to determine water content and deuterium content for 18 samples of the Mount St Helens dome dacite. These isotope data, the first ever collected from an active lava dome, suggest a steady-state process of magma evolution combining crystallization-induced volatile production in the chamber with three different degassing mechanisms: closed-system volatile loss in the magma chamber, open-system volatile release during ascent and kinetically controlled degassing upon eruption at the surface. The data suggest that future dome-building eruptions may require a new influx of volatile-rich magma into the chamber.

Hydrogen isotope analyses of samples from Mount St Helens can be used to investigate the role of changing water content and isotopic composition in eruption processes because both the bulk chemistry and crystallinity have remained essentially unchanged throughout the growth of the dome and because only small amounts of hydrous minerals are present in the lava³. We collected surface samples from various positions on the May 1985 and May 1986 lobes and also obtained samples from seven earlier lobes collected by D. Swanson, K. Cashman and C. Heliker of the U.S. Geological Survey (Table 1). The texture of each sample and the date and position relative to the vent and flow front are given in Table 1. There are two distinct lava textures at Mount St Helens. Smooth lava has <30% bubbles

compared to >50% for scoriaceous lava, which tends to occur as a 2–3 m thick carapace⁴.

Crushed samples were heated to 1,000 °C for 3 h and the water content (Table 1) measured with a manometer (0.002 wt % uncertainty—S. Roberts, personal communication). Hydrogen was produced by the reaction of the expelled water with uranium. The uncertainty in the δD_{SMOW} values is 2‰ (δD_{SMOW} is $\{[(^2\text{H}/^1\text{H})_{\text{sample}}/(^2\text{H}/^1\text{H})_{\text{SMOW}}] - 1\} \times 10^3$ where SMOW is standard mean ocean water). For comparison, meteoric waters taken from springs in the Mount St Helens area have δD values ranging from –82.3 to –111.4‰, and water from a pond in the summit crater in March 1980 had a δD value of –89.8‰ (ref. 5).

One purpose of sampling several different lobes was to identify temporal trends in water content and isotopic character. We found large variations within individual extrusions, however, making simple inter-lobe comparisons difficult to interpret. We therefore grouped samples by texture (smooth or scoriaceous) and position on the flow (near vent or flow front) in an attempt to compare lavas that had undergone similar degassing histories. This reclassification into four subsets (vent smooth, front smooth, vent scoria and front scoria) showed several trends: (1) for samples from a given flow position, scoria consistently had lower water contents than smooth lava; (2) for a given texture, flow-front samples had lower water contents than near-vent samples; and (3) smooth lava from near the flow fronts had higher δD values than from near the vent.

We have tried to correlate the δD data in Table 1 to various eruptive parameters^{2,4} such as lobe volume, underlying slope, ratio of endogenous to exogenous growth and repose period. Because we were interested in processes occurring at depth, we concentrated on smooth samples, as these had apparently been affected less by degassing at the surface⁴. These comparisons revealed one clear trend (Fig. 1): the longer the interval between eruptions, the more deuterium enriched the smooth lava.

Previous isotope studies of volatiles in silicic lavas^{6–8} have identified three different degassing mechanisms. Under closed-system conditions, most volatiles lost from the magma remain in contact with the magma and so continue to interact with it, leading to a gradual decrease in δD as water content decreases⁶. Open-system degassing occurs when expelled volatiles are able to escape, causing a more rapid drop in δD with water loss^{7,8}. When water contents become extremely low, however, kinetic effects prevent HDO from escaping more rapidly than H_2O , so that there is actually an increase in δD with decreasing water content⁸. In the following scenario, we suggest that all three mechanisms operated during eruption and emplacement of the Mount St Helens dome lavas.

We start with the assumption that between eruptions, the top of the magma body slowly loses volatiles, some of which make

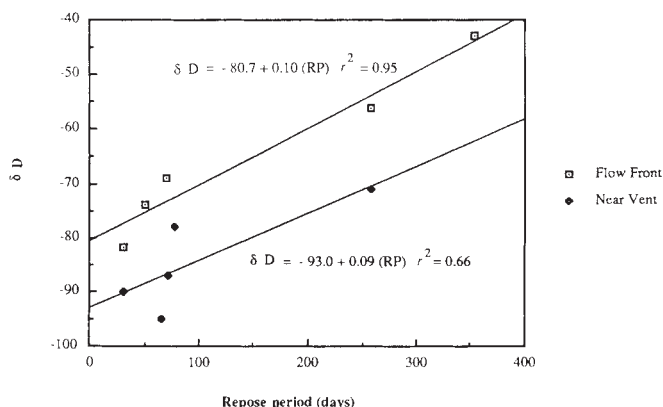


FIG. 1 Plot of δD as a function of repose period for smooth samples from flow-front and near-vent locations. Scoriaceous and mid-flow samples not included.

TABLE 1 Water and isotope data

Extrusion date	Sample #	Texture	Location	H ₂ O content (%)	δD_{SMOW} (‰)
October 1980	SH53	Smooth	Flow front	0.109	-69
October 1980	SH33	Scoria	Near vent	0.161	-60
December 1980	SH61	Scoria	Flow front	0.127	-73
December 1980	SH66	Smooth	Near vent	0.165	-87
February 1981	SH82	Smooth	Flow front	0.081	-82
February 1981	SH69	Scoria	Mid-flow	0.105	-71
February 1981	SH73	Smooth	Near vent	0.212	-90
June 1981	SH91	Scoria	Flow front	0.126	-71
June 1981	SH108	Smooth	Near vent	0.194	-95
September 1981	SH106	Smooth	Near vent	0.157	-78
October 1981	SH109	Smooth	Flow front	0.201	-74
March 1982	SH118	Scoria	Flow front	0.062	-75
May 1985	12HCS85	Smooth	Flow front	0.210	-56
May 1985	2HCS85	Smooth	Near vent	0.230	-71
May 1986	5HCS786	Smooth	Flow front	0.111	-43
May 1986	9HCS786	Smooth	Mid-flow	0.271	-67
May 1986	14VC786	Smooth	Mid-flow	0.337	-40
May 1986	11HCS786	Scoria	Near vent	0.149	-46

their way into the eruptive plume over the dome⁹. Although a small fraction of these volatiles escape from the top of the magma body, we feel that this drying out at depth will follow a closed-system fractionation trend, similar to that proposed for some rhyolitic magmas^{10,11}. We assume that all of the eruptions are ultimately caused by the steady-state production of volatiles during crystallization of anhydrous minerals in the magma^{12,13}. When the resulting buoyancy becomes sufficient, the magma body begins to rise, pushing its dry cap through the conduit to the dome surface. As it works its way upward, the magma loses volatiles more rapidly through fractures in the conduit walls and dome, leading to open-system degassing⁷. When the volatile content has decreased to approximately a few tenths of one percent, kinetically controlled degassing ensues. The extrusion continues until all of the buoyant magma has risen to a new equilibrium configuration. These changes in degassing behaviour cause the δD ratio of any particular magma batch to follow a complex path similar to that represented schematically in Fig. 2.

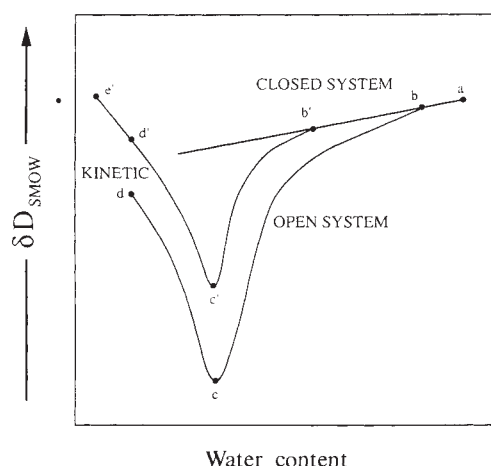


FIG. 2 Schematic diagram showing proposed three-stage degassing curves for two different dome-building eruptions at Mount St Helens. Both batches of magma begin with δD and water contents indicated by point *a*. The first batch has a short repose period and then follows path *b-c-d* as it rises to the surface and erupts. The second batch has a longer repose and then follows path *b'-c'-d'*. Note that the longer hiatus leads to higher δD values for a given water content. Point *e'* represents a hypothetical sample of smooth lava collected from the flow front of the second extrusion which would have travelled further at the surface than a smooth lava sample from near the vent area, *d'*.

The slopes of the closed- and open-system fractionation paths in Fig. 2 are arbitrary, because the fractionation factors are not known for Mount St Helens dacite and we also have no data on which to base a starting point. We expect that these factors would depend only on magma composition, however, and thus would not have changed appreciably during the six-year emplacement of the dome. Similarly, the critical water content below which kinetic effects appear is probably independent of time. Thus if different batches of magma had different repose periods before they began their ascent, they subsequently would follow roughly parallel curves on a plot of δD as a function of H₂O.

Consider the magma body before two different eruptions, as depicted by the curves on Fig. 2. In both cases, when the preceding eruptive episode ends, the water content and δD values of the magma are represented by point *a*. Each batch then follows the closed-system degassing path until buoyancy causes the magma to rise. In the first case this point *b* is reached after a hiatus of 50 days, whereas the second batch takes 150 days and enters the open-system portion of the path having a lower water content *b'*. During ascent, the first batch will reach the point *c*, at which kinetic effects begin, with a lower δD value than the second batch *c'*. If both come to rest in the same dome position (near the vent for example), then they will have undergone similar amounts of kinetically controlled degassing, and the magma with the longer repose period will have a higher δD value *d'*. Within each batch, the portion that reaches the flow front *e'* will have degassed longer and would thus have a higher δD (and lower water content) than the near-vent portion *d'* (ref. 4).

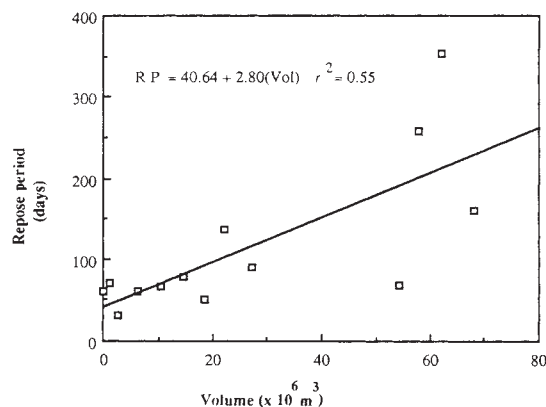


FIG. 3 Repose period as a function of dome volume ($\times 10^6 \text{ m}^3$) for Mount St Helens extrusions. Volumes used² are those of the dome before a given repose period. Volume before October 1980 is assumed to be zero.

Our model accounts for observed δD variations, but does not explain why repose periods vary. If we compare repose duration with dome volume, however, we find a fairly good correlation (Fig. 3) which suggests that as the dome grows, more buoyancy is needed to overcome the increased lithostatic load. Thus if eruptions are driven by a steady-state process such as crystallization-induced volatile production, the greater weight associated with an increase in dome volume will require a longer time for the critical buoyancy to be generated. Other predictable factors such as earth tides¹⁴ or melting of snowpack in the crater¹⁵ can facilitate the eruption process but by themselves are of insufficient magnitude to serve as triggers. On the other hand, the influx of new volatile-enriched magma into the chamber from a deeper source, as is thought to have occurred in March 1982¹⁶, could enhance the buoyancy and lead to a new eruption.

This view of dome growth allows certain predictions to be made about future activity. If the trend shown in Fig. 1 were continuing today and a new eruption were to begin in October 1989, for example, then the three-year (1,095 days) hiatus would suggest that the smooth lava that came out and reached the flow front would be extremely enriched in deuterium ($\delta D \approx 30\%$). At the same time, the volume of the dome after the October 1986 eruption indicates that the following hiatus should have been around 230 days, leading to an eruption in June 1987. The failure of magma to appear at that time suggests that some other factor was involved, such as the completion of crystallization or the development of a yield strength in the steadily drying magma body. The resumption of eruptive activity may require the influx of a new batch of volatile-rich or crystal-poor magma. □

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Undercrusting by serpentinite beneath rifted margins

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THE west Galicia passive margin (Spain) is bounded by a continuous belt of serpentinitized peridotite. Here we present the results of petrological studies of the peridotite, ^{39}Ar – ^{40}Ar dating of high-temperature ductile shearing of a dioritic dyke crossing the peridotite and regional seismic stratigraphy which demonstrate that the uplift, ductile shearing and pervasive serpentinitization of mantle rocks occurred at early Cretaceous time, during the rifting stage of the margin. The serpentinite layer resting on fresh peridotite is probably several kilometres thick. At present seismic data and gravity data cannot distinguish serpentinite from crustal material, but our results from the Galicia margin indicate that the seismic crust beneath rifted margin may include mantle material transformed into serpentinite by syn-rift hydrothermal activity. In some cases, this undercrusting process might explain the young age and mobility of the Moho beneath stretched continental basement.

The west Galicia margin, Spain (Figs 1, 2) is a passive margin, formed during a Berriasian/latest Aptian rifting episode (140–114 Myr BP). Recently, it was investigated by DV *JOIDES Resolution* (Ocean Drilling Program Leg 103)^{1,2} and the French submersible *Nautilus* (Galinaute cruise)³. Westward, at the continent-ocean boundary in the Atlantic Ocean, the basement beneath the sediments forms a ridge (Fig. 2) surveyed by reflection seismics⁴ for ~130 km (Fig. 1). At its southern part, the ridge strikes N–S parallel to the trend of the tilted fault blocks of the margin. North of 43° N, it turns eastward along the northwestern edge of the Galicia Bank. Its eastern flank is partly

buried under lower Cretaceous syn-rift sediments (Fig. 3), indicating that it had formed before sea-floor spreading started.

The ridge consists of highly serpentinitized (up to 90%) peridotite, at places replaced and crosscut by calcite veins^{5–10}. The peridotites are mylonitic spinel- and plagioclase-bearing harzburgites or lherzolites that underwent limited melt extraction (<10%)^{6,7}. At dive site 10 (Fig. 1), the ultramafic is crosscut by a several-centimetres-thick dioritic dyke, containing plagioclase, brown amphibole and ilmenite. This dyke, as well as the peridotite, underwent ductile shearing at high temperatures (>750 °C) and large differential ($\sigma_1 - \sigma_3$) stresses (>1 kbar), and now displays a mylonitic foliation sub-parallel to that observed in the surrounding rock⁹. In the diorite, all the primary magmatic brown amphiboles have recrystallized into millimetre-sized grains during the shearing process. Some crystals, ~3 cm in size, however, grew at the expense of the fine-grained neoblasts at the end of the shearing.

Using the ^{39}Ar – ^{40}Ar method we dated large post-tectonic crystals of brown amphibole¹¹, fine-grained syn-tectonic amphibole and syn-tectonic plagioclase neoblasts. The post-tectonic amphiboles provided a plateau age of 122 ± 0.3 Myr (Fig. 4), corresponding to a constant K/Ca ratio (proportional to $^{39}\text{Ar}_K / ^{37}\text{Ar}_{Ca}$) similar to the microprobe-measured ratio. The fine-grained amphibole provided a scattered age spectrum (see Fig. 4) with concordant high-temperature ages that are nearly consistent with the plateau age of the post-tectonic amphibole. The plagioclase has a saddle-shaped age spectrum which is characteristic of excess argon. The weighted mean of 117.7 ± 0.9 Myr (calculated on the 700–1,080 °C steps) may represent a maximum estimate. Although the plagioclase crystallized before the post-tectonic amphibole, these results are concordant with the cooling history of the diorite, the closure temperature below which all radiogenic ^{40}Ar is trapped within the mineral of the amphibole being higher than that of the plagioclase. We therefore conclude that the 122.0-Myr age corresponds to the end of the ductile shearing of the dioritic dyke. Because the ductile deformation of the dioritic dyke and that of the surrounding peridotite occurred under a similar strain regime and physical conditions, we consider that this age is also that of the emplacement of the peridotite near the surface of the lithosphere¹¹.

This result indicates that uplift and emplacement of ultramafic

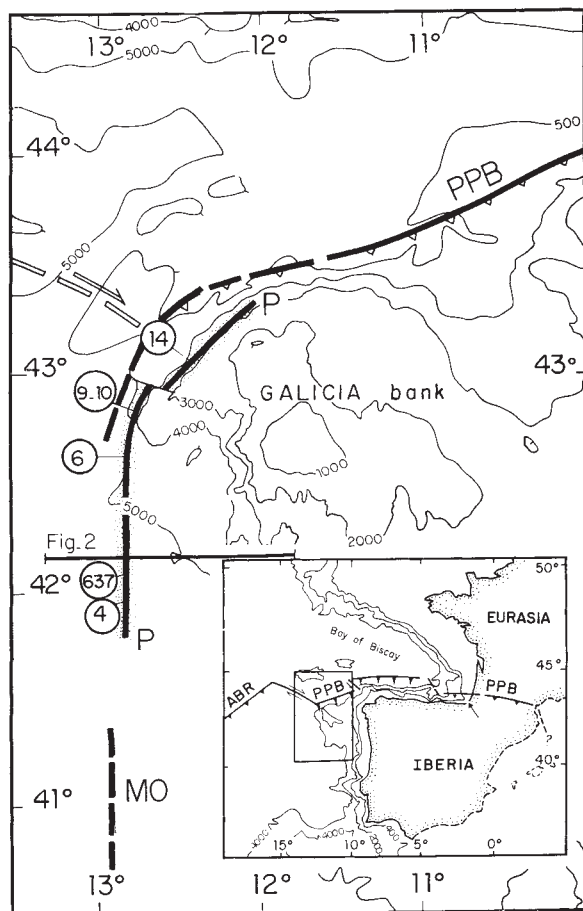


FIG. 1 Location of drill site 637 (ODP Leg 103^{1,2}) and dive sites 4, 6, 9–10 and 14 (Galinaute cruise³) where peridotite was sampled on the Galicia margin. Bathymetry in metres. MO, MO magnetic anomaly²²; ABR, Azores-Biscay Rise; P, Peridotite ridge; PPB, Palaeocene plate boundary²³.

rocks occurred during the rifting of the margin (140–114 Myr), before the opening of the north Atlantic Ocean between the Iberian and American plates. As the serpentinite is broken by normal faults^{7,8} and sealed by syn-rift sediments⁴, we assume that most of the serpentinitization of the peridotite also occurred during rifting, probably at the latest stage of emplacement of mantle rocks on the sea floor, as a result of the intrusion of sea water within the rock⁸.

At present, there are only few other examples of serpentinite belts bounding rifted margins (south Australia margin¹²; Tyrrhenian sea¹³). Usually, the ocean-continent transition is buried under thick sediments preventing from drilling into basement. The west Galicia margin is exceptional in that it is a very starved margin with only a thin and discontinuous sedimentary cover above basement. It is, however, a very typical rifted margin and we assume that the peridotite ridge is representative, although

other kinds of ocean-continent transition can occur in the global ocean.

Unfortunately, as seismic refraction data are not yet available on the Galicia margin, the thickness of the serpentinite layer that overlies fresh peridotite is poorly constrained, but it can be roughly estimated. Therefore no noticeable anomalies in a gravity survey of that region¹⁴, so the hypothesis of local isostatic balance is reliable. The ocean is 5,250-m deep, and the sediment thickness ranges from 0 to 2.5 km (ref. 4) assuming a mean density of 2.2 g cm^{-3} . Petrological studies of drilled samples have shown that serpentinitization is inhomogeneous and varies from 100% to 60%^{6–8}. Accordingly, density would vary from 2.6 to 2.9 g cm^{-3} (ref. 15). In our opinion, a mean density of 2.9 g cm^{-3} seems to be realistic, as serpentinitization is expected to decrease at depth. On the other hand, 3.35 g cm^{-3} is a reliable value for the density of fresh peridotite from old lithosphere (>114 Myr). Then, the calculated serpentinite thickness at the ocean-continent transition of the west Galicia margin ranges from ~ 9 km to ~ 2.5 km, depending on sediment thickness.

This estimate is not surprising: it is now accepted that sea water can penetrate the upper lithosphere down to several kilometres, and the breakdown of olivine into a serpentinite assemblage¹⁶ is usual at the pressure and temperature conditions expected during rifting beneath the sediments covering the peridotite ridge or beneath the much thinned and faulted continental crust of the deep Galicia margin as well.

Figure 2 shows the possible eastward continuation of the serpentinite layer from the peridotite ridge to the deep margin. This lateral extent may be related to the strong seismic reflector, labelled S, that occurs within the basement of the deep margin. On average, S extends at 5–2 km depth below the layered sedimentary cover⁴, deepens landward and seems to intersect the top of the basement between the peridotite ridge and the deepest continental block of the margin¹⁷ (Figs 2 and 3). It may be the seismic signature of the contact between the serpentinite and the continental basement, a place we expect an impedance contrast.

On the west Galicia margin, it seems that: (1) the stretching of the lithosphere during the rifting stage of the margin may result in total removal of the continental crust and unroofing of the mantle at the continental rift axis; (2) serpentinitization of the exposed mantle peridotite can occur at several kilometres depth below the sea floor, and the serpentinite layer may extend laterally beneath the very thinned and faulted continental crust of the rift. (3) Serpentinite has a density¹⁵ and seismic velocity¹⁸ comparable to that of the lower continental crust and therefore neither seismic refraction nor the gravity data can be used to distinguish definitely between lower continental crust and serpentinitized upper mantle material. However, we think, by analogy with seismic crust of oceanic lithosphere, that the seismic crust of stretched continental lithosphere may comprise serpentinite generated by hydrothermal alteration of the uppermost mantle. We call this process undercrusting to distinguish it from underplating, which relates to upward magma crystallization at the crust-mantle boundary during rifting¹⁹. Indeed, undercrusting is probably not as important as underplating, as volcanic rocks in continental areas do not often contain serpentinite xenoliths. However, we think that it can play a significant

FIG. 2 Synthetic cross-section of the deep Galicia passive margin¹⁷ (location on Fig. 1). The Moho depth is calculated assuming isostatic equilibrium, densities of 2.8 g cm^{-3} for the thinned continental crust, 2.9 g cm^{-3} for serpentinite and 3.35 g cm^{-3} for fresh peridotite. M, seismic Moho; S, S seismic reflector²⁴ assumed to be the contact between serpentinite and thinned continental crust. S was faulted at the latest stage of the rifting.

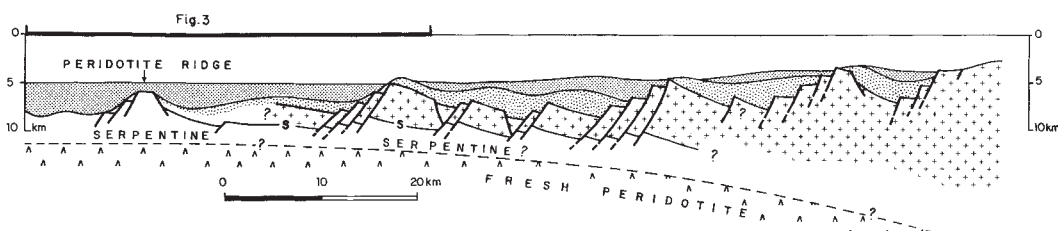
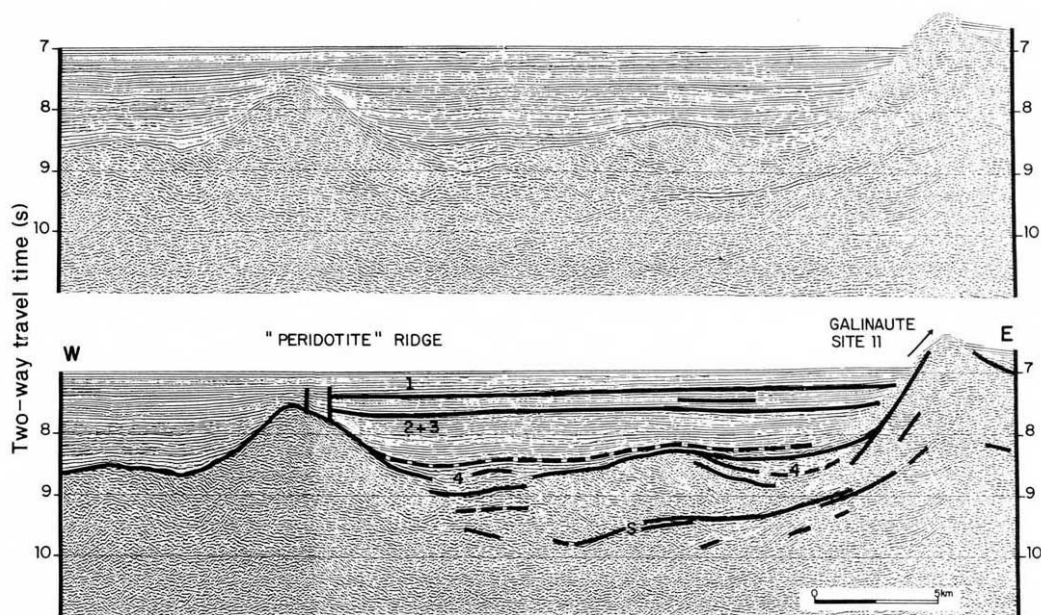


FIG. 3 Seismic line⁴ depicting the peridotite ridge and the deepest fault block of the west Galicia margin, where the granodioritic continental basement was sampled³. S, S seismic reflector²⁴. 1–3, post-rift sediment; 4, syn-rift sediments. See Fig. 2 for location.



part beneath advanced continental rifts. Such an interpretation is supported by the existence of the regional seismic reflector S, mentioned above. (4) Wide-angle reflections at the base of the continental crust indicate that the reflective Moho is relatively young with respect to the age of the upper continental basement and even to several Mesozoic basin formations as well^{20,21}. Perhaps this is a consequence of the downward migra-

tion of a hydrothermal front and the subsequent serpentinization of fresh peridotite at the top of the mantle, as probably occurred beneath the Galicia continental rift in the Cretaceous. We do not yet know the greatest depth to which water may be introduced in the continental lithosphere and whether layering of lower continental crust may result from inhomogeneous serpentinization of mantle rocks. Lenses of poorly serpentinized peridotite entrapped within highly serpentinized material could account for seismic reflectors at the base of the continental crust in rifted zones. Our suggestions, although based on the unique example of the West Galicia margin, raise important questions relevant to both the mobility of Moho beneath stretched continental basement and the actual nature of the lower continental crust. □

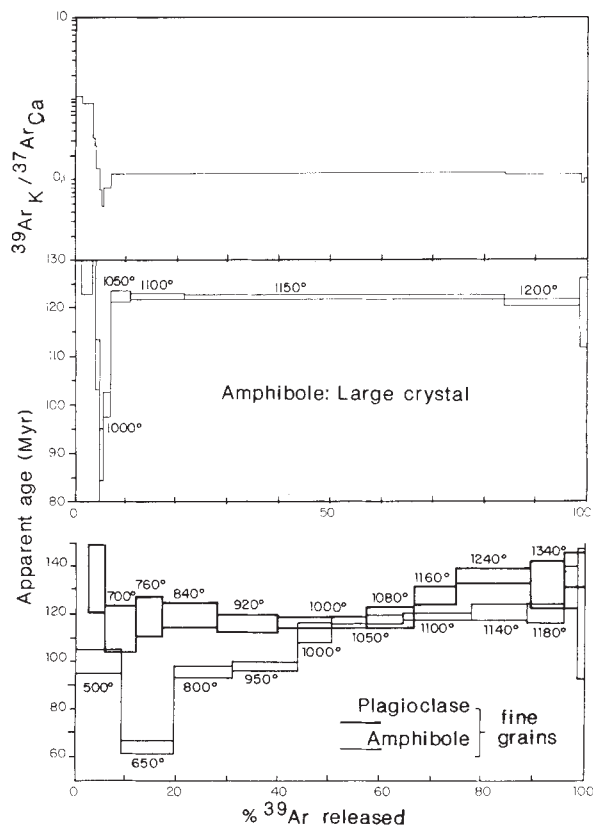


FIG. 4 ³⁹Ar–⁴⁰Ar age spectra for euhedral undeformed amphibole, fine-grained amphibole and plagioclase sampled in a dioritic vein crossing peridotite at dive site 10. See Fig. 1 for location. Samples were irradiated in the Melusine reactor (CEN Grenoble). The monitor was the biotite LP6 128.5 Myr in age. Uncertainties are at the 1σ level.

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The oxidation state of the Earth's sub-oceanic mantle from oxygen thermobarometry of abyssal spinel peridotites

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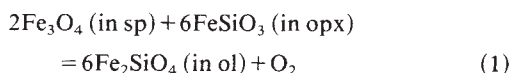
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THE oxygen fugacity (f_{O_2}), or redox state, of the Earth's mantle is an important parameter in the evolution of mantle-derived magmatic rocks. Most estimates of the upper-mantle redox state have been based on samples of the sub-continental mantle and terrestrial lavas, and estimates of f_{O_2} obtained by thermobarometric methods tend to cluster within ± 1.5 log units of the FMQ (fayalite-magnetite-quartz) reference oxygen fugacity buffer¹⁻³. Much less is known about the redox state of the sub-oceanic mantle, the ultimate source of mid-ocean-ridge basalts (MORBs). Here we use oxygen thermobarometry of abyssal spinel peridotites, representative of most of the Earth's mid-ocean-ridge systems, to show that the redox state of the sub-oceanic mantle lies at values of up to 4 log units (average of 1.35 log units) more reduced than FMQ. Our results are in excellent agreement with oxygen fugacities of MORBs obtained from Fe^{3+}/Fe^{2+} ratios of quenched glass in pillow basalts⁴. This agreement confirms the redox state of the MORB source region and suggests that MORB glasses (as opposed to the cores of pillow basalts) have not undergone significant oxidation (hydrogen degassing) during their ascent.

We have sampled abyssal spinel peridotites ($n=33$) from fracture zones along the Central Indian, America-Antarctica, South-west Indian and Mid-Atlantic Ridge systems as well as the Cayman Trough. Although variably affected by hydrothermal alteration, all of our samples contained fresh spinel (sp) and olivine (ol), with most samples also containing orthopyroxene (opx); they are, therefore, amenable to application of the spinel-peridotite oxygen thermobarometer¹⁻³.

Oxygen thermobarometry is based on the heterogeneous equilibrium



Fe_2SiO_4 refers to the fayalite component in olivine, $FeSiO_3$ the ferrosilite component in orthopyroxene, and Fe_3O_4 refers to the magnetite component in spinel. The thermodynamic properties of complex $MgAl_2O_4$ - Fe_3O_4 spinels have recently been calibrated¹ for the temperature range 900–1,100 °C and spinel compositions common to upper-mantle spinel lherzolites ($0.01 \leq X_{Fe_3O_4}^{sp} \leq 0.10$). As long as compositions of all phases are known, together with appropriate activity-composition relationships, it is possible from available standard-state data to obtain estimates of f_{O_2} at any temperature and pressure^{3,5}. Silicate-mineral compositions were obtained by electron-microprobe analysis⁶. Mössbauer-analysed natural spinels of widely variable Cr number ($Cr/(Cr+Al)$) and $Fe^{3+}/\Sigma Fe$ ratios were used as secondary electron-microprobe standards for spinels. To avoid any possible effects of submarine hydrothermal alteration on the silicate phases, all mineral analyses were made on fused glasses of hand-picked optically clear diopside and enstatite, to homogenize solution lamellae (where present) and to determine a primary composition for these phases⁶. Mineral analyses were also performed on optically clear hand-picked grains of olivine.

We obtained spinel concentrates by digesting samples of abyssal spinel peridotite in 50% (wt%) HF acid, followed by several digestions in 35% (wt%) HCl acid. This ensured the removal of any magnetite-haematite alteration of the spinel. All spinel concentrates were then passed through a Frantz magnetic separator. No magnetic material remained after the HCl-acid treatment and optical examination under reflected light revealed that only a pure spinel concentrate remained. We obtained magnetite contents of spinel by microprobe analyses and also by Mössbauer spectroscopy on the clean HCl-treated samples using the methods described in ref. 3. Mole fractions of Fe_3O_4 in spinels from abyssal peridotites range between 0.006 and 0.047 with a mean of 0.021. The microprobe and Mössbauer methods agree with one another, generally giving results within ± 0.003 in $X_{Fe_3O_4}$.

We obtained temperatures of equilibration using an empirical two-pyroxene geothermometer⁷. Not all of our abyssal peridotite samples contained two pyroxenes suitable for geothermometry. For these samples, a mean temperature estimate based on a suite of eleven samples from the America/Antarctica/South-west Indian Ridges was used ($1,153 \pm 75$ °C; $n=11$, ± 1 s.d.). We assumed the fayalite and ferrosilite contents of samples lacking either olivine and/or orthopyroxene to be 0.09, also based on average compositions of these phases in the same suite of eleven abyssal peridotite samples.

We measured oxygen fugacities of abyssal peridotites using equilibrium (1) at an estimated total pressure of 10 kbar. As there is no presently available means of estimating equilibration pressures for spinel peridotites a pressure of 10 kbar was chosen primarily for two reasons. First, the rocks for which we report results here are plagioclase-free spinel peridotites. Second, plagioclase-bearing peridotites have also been reported⁶ to occur at several localities along the mid-ocean ridges that we have also sampled. A pressure of 10 kbar at 1,153 °C is in the spinel-peridotite field of stability but also close to the boundary between plagioclase-bearing and spinel-bearing peridotites. The results have been plotted in Figs 1 and 2, as $\Delta \log f_{O_2}$ relative to the calibration of the FMQ-reference f_{O_2} buffer⁸, $\Delta \log f_{O_2}^{FMQ}$. Significantly, almost all of the f_{O_2} data are values more reducing than FMQ. Another important feature of the abyssal-peridotite f_{O_2} data is that they seem to be bimodally distributed, with over 80% of our samples lying between $\Delta \log f_{O_2}^{FMQ} = -2.5$ and +1,

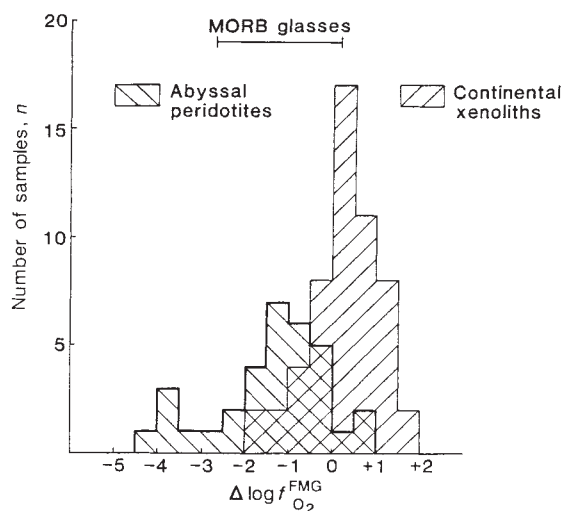


FIG. 1 Calculated $\Delta \log f_{O_2}$ distributions (relative to FMQ) for spinel-bearing abyssal peridotites (this study) and sub-continental mantle spinel lherzolites (unpublished data and ref. 2). The majority of abyssal peridotites lie at f_{O_2} conditions more reduced than FMQ compared to mantle spinel lherzolites which form a tight distribution (± 1.5 log units) at FMQ. These data suggest that the sub-oceanic mantle is more reduced than the sub-continental mantle. f_{O_2} data for MORB glasses is from ref. 4 and defines a range in $\Delta \log f_{O_2}$ in excellent agreement with the abyssal peridotite values.

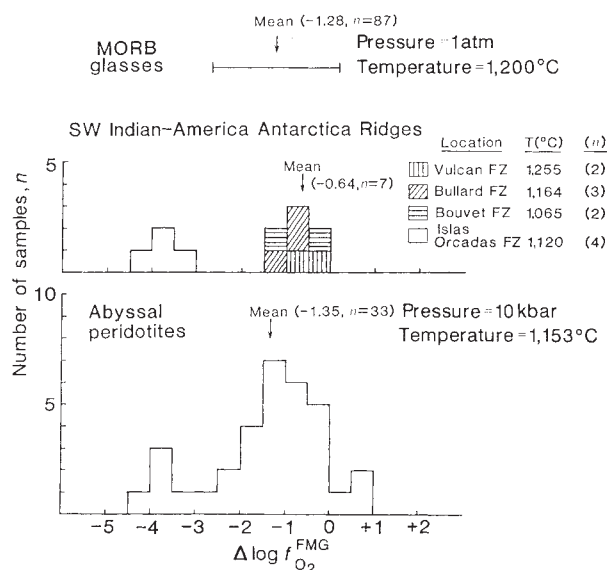


FIG. 2 $\Delta \log f_{O_2}$ (relative to FMQ) results for abyssal peridotite samples from the America/Antarctica/South-west Indian Ridges in the vicinity of the postulated Bouvet Island hotspot. Note the anomalously reduced nature of abyssal peridotites from the Islas Orcadas fracture zone (FZ) relative to all other samples.

and the remainder being distributed almost exactly at the iron-wüstite (IW) reference buffer ($\Delta \log f_{O_2}^{FMQ} \approx -3.75$). Four of the six samples close to IW belong to a suite of samples collected from the Islas Orcadas fracture zone near the mantle hotspot present^{9,10} in the Bouvet Island area. The results in Fig. 2 suggest that the mantle near the hotspot may be anomalously reduced compared to the mantle beneath mid-ocean ridges in general. Our results show that the upper mantle beneath mid-ocean ridges has a mean $\Delta \log f_{O_2}^{FMQ}$ of -1.35 ± 1.3 ($n=33$; ± 1 s.d.) with values ranging from near FMQ to IW.

The range of f_{O_2} values observed in continental spinel lherzolites is also given in Fig. 1. Comparison of the two data sets shows that both the mean and range of f_{O_2} values observed in abyssal spinel peridotites are significantly more reduced than those previously reported for continental spinel lherzolites¹⁻³. Our results are, however, in very good agreement with f_{O_2} values reported⁵ for continental spinel lherzolites from eastern Australia and the south-west United States, based on a different calibration of a spinel oxygen thermobarometer⁵. These results imply a possible genetic link between abyssal spinel peridotites and some suites of continental spinel-lherzolite xenoliths as has recently been suggested³. Our data also show that the sub-oceanic mantle is more reduced than has been suggested¹¹⁻¹³. Values near IW (ref. 11) were obtained by the intrinsic zircon-cell technique on two samples of peridotite. The intrinsic f_{O_2} measurements have recently been shown to be unreliable and in general give f_{O_2} values considerably more reduced than are obtained using other techniques¹⁴.

The redox states of a large ($n=87$) and geographically diverse suite of MORBs showed⁴ that the Fe^{3+}/Fe^{2+} ratios of pillow cores give f_{O_2} values in the range FMQ–NNO (nickel/nickel oxide f_{O_2} buffer) in agreement with subaerial basalts. Fe^{3+}/Fe^{2+} ratios of rapidly quenched glasses from the pillow margins, however, indicated f_{O_2} values 1 to 2 log units below FMQ. These results suggested that the MORB source regions are more reduced than FMQ and that lavas must oxidize rapidly during cooling, possibly by loss of hydrogen. The MORB data of ref. 4 therefore placed constraints on the upper limit for the redox state of the MORB source region. Those results have been plotted on Figs 1 and 2 to facilitate comparison with the abyssal peridotite data. The mean $\Delta \log f_{O_2}^{FMQ}$ is -1.28 ± 0.63 ($n=87$;

± 1 s.d.), a result in excellent agreement with our thermobarometric estimate for the redox state of the MORB source region. The agreement between the f_{O_2} values indicated by MORB glasses and the abyssal spinel peridotites suggest to us that the MORB glasses (as opposed to pillow cores) have not undergone any significant oxidation or degassing. Hence, we conclude that the data from abyssal spinel peridotites confirms the redox state of the sub-oceanic mantle and the MORB source region as determined in ref. 4. There is considerable uncertainty as to whether pressure has a significant effect on the Fe^{3+}/Fe^{2+} ratio¹⁵⁻¹⁷ and the f_{O_2} that such a ratio represents. Because of the uncertainties associated in estimating the $\Delta \log f_{O_2}$ variations as a function of pressure-related changes in Fe^{3+}/Fe^{2+} ratios, we prefer, at present, to accept the results of experiments that were conducted with basaltic liquids and in which f_{O_2} was a controlled variable¹⁵, a condition not satisfied by the other studies^{16,17}. The pressure effect on the Fe^{3+}/Fe^{2+} ratio in basaltic liquids (1 bar–10 kbar) is negligible¹⁵. We have therefore made no pressure correction to the f_{O_2} values indicated by the MORB glasses in Figs 1 and 2. The agreement between f_{O_2} estimated for the MORB glasses and abyssal spinel peridotites may be taken to indicate that the pressure effect on Fe^{3+}/Fe^{2+} ratios, if real, is quite small, consistent with ref. 15. □

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Record of Palaeozoic pseudoscorpions

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PSEUDOSCORPIONS (Class Arachnida; Order Pseudoscorpiones) represent a diverse and abundant group of small predatory arthropods¹⁻⁴. Approximately 3,000 living species occupy habitats ranging from sea level to 5,000 metres elevation. Until now, the fossil record of the group extended back only 35 million years, to the Oligocene, where nearly 30 species, all in extant families, have been described from the Baltic Amber⁵. Addition pseudoscorpions have been described from the younger Dominican⁶ and Mexican⁷ ambers, and they too are all assignable to modern families. We now report the discovery of two pseudoscorpion specimens in Middle Devonian sediments near Gilboa, New York, USA. This

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find increases the documented age of the order more than 10-fold to ~380 million years. Although the specimens cannot be assigned with certainty to any extant superfamily, their excellent state of preservation allows us to make numerous inferences concerning behaviour and ecology, such as silk use, grooming and tactile behaviour. The presence of certain well preserved structures indicates that Devonian pseudoscorpions were predatory, as are their modern counterparts. Our discovery provides further evidence that fully terrestrial arthropods coexisted with primitive vascular plants during the Middle Devonian.

Middle Devonian sediments near Gilboa in New York State have yielded an abundant and diverse fauna of terrestrial arthropods⁸, including extinct groups such as trigonotarbid⁹ and arthropleurids, as well as the earliest representatives of groups still living, including centipedes¹⁰, oribatid mites¹¹ and spiders¹². To the latter list we can now add pseudoscorpions; the discovery of two specimens of this order increases the documented age of the group by more than 10-fold, to ~380 million years. Significantly, the Devonian fossils already possess all major apomorphies of the order, but in a combination not known from any surviving clade of pseudoscorpions. As with the other Gilboa fossils, the two pseudoscorpions were extracted by hydrofluoric acid maceration from a black shale rich in plant remains. The appearance of the fossils, their authenticity, and the methods by which they are studied have been discussed in detail⁹.

The more complete of the two specimens (Fig. 1) is ~0.76 mm in its widest dimension, and 0.45 mm long. The specimen includes both chelicerae, one complete and one partial pedipalp, both first legs and parts of other legs, and the first four or five abdominal segments. The excellent preservation has allowed us to see such details as the many specialized structures of the chelicera (see below), the distribution of trichobothria on the palpal chela, and the pattern of jointing of at least the first leg, all important for systematic assignment.

The second specimen is from a larger animal and consists only of a single pedipalp chela and a single chelicera; the specimen is ~0.72 mm long, indicating that the animal from which it came would have been about twice the size of the first specimen. If anything, the preservation of this specimen is better than that of the first, allowing at least the palpal chela and the chelicera to be fully compared with the same structures of living species.

The complex adaptations of the chelicerae of pseudoscorpions are characteristic of the order¹ and are all clearly present in the fossils. The chelicera consists of two parts, a large basal one with a fixed finger, and a distal one which acts as a movable finger. In nearly all pseudoscorpions, including the Devonian

fossil forms, the ventral surface of the basal part bears a group of modified setae called a flagellum (f in Fig. 3); the function of this structure is not known. The fixed cheliceral finger exhibits a broad, transparent toothed structure known as the serrula interior (si in Fig. 3), used in grooming. Ventral on the movable finger is a similar structure, the serrula exterior (se in Fig. 3), also for grooming and cleaning the other appendages⁴. The movable finger has an apical galea (g in Fig. 3), or spinneret, which, when present in living forms, produces silk used to construct moulting and resting chambers⁴.

The pedipalps of pseudoscorpions are chelate, an adaptation which causes them to resemble true scorpions, to which they are only distantly related¹³. In many living clades of pseudoscorpions, venom glands are present in one or both chelal fingers; we could not detect a venom apparatus in either of the fossil specimens. The pedipalps are also important sensory organs. Pseudoscorpions have small eyes which are presumably of little use in their dark habitats, and locate prey primarily by touch or by the perception of airborne vibrations³. These stimuli are picked up by highly modified sensory hairs on the pedipalps called trichobothria. Trichobothrial hairs, extremely thin and delicate, have not been preserved on the fossils, but the bothria, the characteristic sockets from which the hairs extend, are present (b in Fig. 2). The distribution of these sense organs is of great significance in pseudoscorpion systematics^{1,2}, and in the study of their postembryonic development¹.

The location and number of palpal bothria indicates that both our specimens represent juvenile instars, and that the smaller may be a first post-hatching, or protonymphal, instar, because it has only a single bothrium on the movable finger of the chela. The larger specimen, with three bothria, may be a last-instar juvenile, or tritonymph, one moult preceding sexual maturity¹. Though modern pseudoscorpions have four post-hatching instars⁴, we of course do not know if this was the case in the Devonian. We also do not know if our specimens represent moulted cuticles or actual carcasses, but the delicate nature of the material indicates the former.

Because the specimens are obviously from different instars, we can express no opinion on their conspecificity. As to further systematic placement, Table 1 compares characters of both fossils (not all the listed characters can be seen on each specimen) with what we believe to be the two pseudoscorpion superfamilies most likely to be related to the fossils, Chthonioidea and Neobisioidea. We have selected these two superfamilies because of the large chelicerae, two-segmented anterior leg tarsi, and smooth cuticle of the fossils⁴. On the nine listed characteristics, the fossils agree with Chthonioidea in two, with Neobisioidea in five, and differ from both in two instances. If

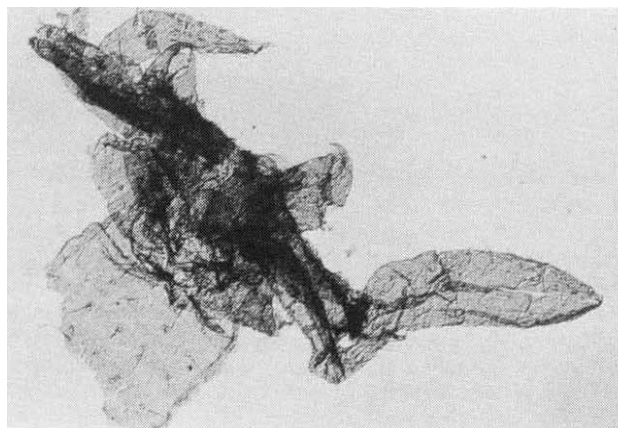


FIG. 1 Specimen of pseudoscorpion protonymph from the Middle Devonian of Gilboa, New York. Slide number 411-19-AR17. Photographed at $\times 100$ on Kodak Technical Pan film using Nomarski differential interference contrast.

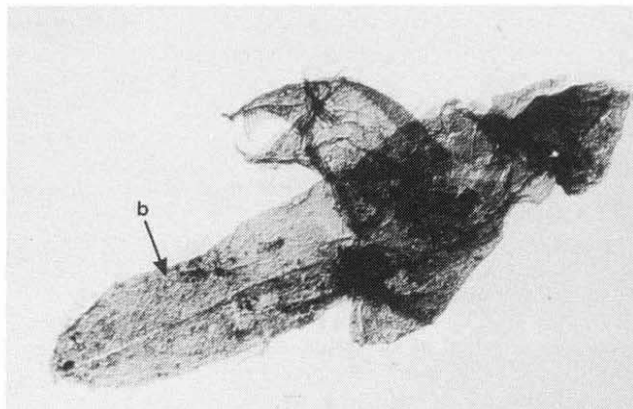


FIG. 2 Palpal chela and chelicera of pseudoscorpion tritonymph from the Middle Devonian of Gilboa, New York. Slide number 411-19-AR9. Method as for Fig. 1; b, bothrium (see text).

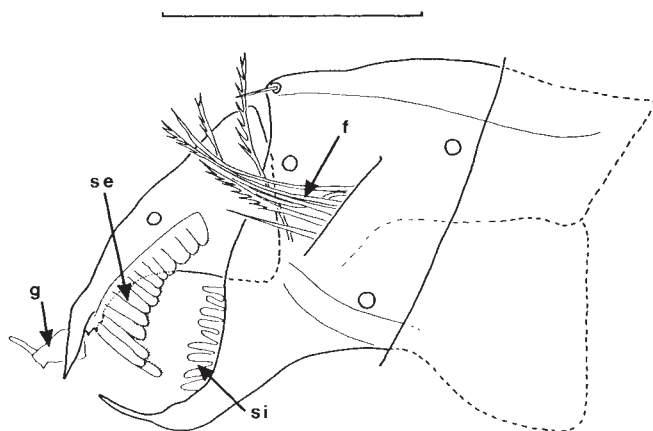


FIG. 3 Interpretive drawing of tritonymph chelicera. Key: f, flagellum; g, galea; se, serrula exterior; si, serrula interior. Scale bar, 0.1 mm.

the larger fossil is indeed a tritonymph, it already has a larger number of fixed finger bothria (six plus two) than the adult of any known living species. Smooth serrula interior blades are found in adults of some living species, but to our knowledge, not in any adult chthonioids or neobisioidea. More detailed study of these specimens, now in progress, may reveal further details clarifying their relationship to living forms.

Cladistic methods have yet to be applied to the study of pseudoscorpion phylogenetics, so we are not able confidently to assign polarities to any of these characters. But the Devonian fossil pseudoscorpions probably cannot be placed in an extant superfamily.

The preservation of details in the fossils also allows us to make some inferences about the autecology and behaviour of Devonian pseudoscorpions. The presence of a galea establishes this early date for silk use by the group. Devonian pseudoscorpions also groomed themselves using cheliceral serrulae. The structure of the palpi and chelicerae tells us that Devonian pseudoscorpions were predators, like their modern relatives. But they may not have had a palpal venom apparatus. A rich complement of palpal trichobothria is significant, because it means that touch and airborne vibrations were important to the Devonian species, and that they were fully terrestrial, because trichobothria cannot function in water¹⁴. We were not able to

find spiracles, which normally occur on the third and fourth sternites, in the more complete of the two fossils. The thin, ungranulated cuticle of the specimens is characteristic of modern pseudoscorpions from humid biotopes.

It is significant for arachnid evolution that the existence in the Devonian of modern-looking pseudoscorpions also implies that their sister group, the Order Solifugae¹³, was also present. The single known Palaeozoic solifuge is from the Upper Carboniferous of Mazon Creek, Illinois, USA¹⁵.

In the larger scheme of the evolution of terrestrial arthropods, these specimens are important because they establish, as has already been done by Gilboa fossils for centipedes¹⁰, two major clades of mites^{11,16}, and spiders¹², that fully terrestrial arthropods of modern aspect coexisted with the primitive vascular land plants of the Middle Devonian. Arthropods may have preceded vascular plants on to land, and algal or cyanobacterial mats may have served as major primary producers in the early evolution of terrestrial ecosystems. □

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A possible neuronal basis for Doppler-shift compensation in echo-locating horseshoe bats

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THE auditory system of the horseshoe bat is finely tuned to the bat's individual vocalization frequency^{1,2}. To compensate for flight-induced Doppler shifts in the echo frequency, the horseshoe bat adjusts the frequency of its echo-location call to maintain the echo frequency within the narrow range to which its auditory system is best tuned^{3,4}. In this report I describe neurons in the midbrain tegmentum of the horseshoe bat, with properties that strongly indicate their involvement in this Doppler-shift compensation. The activity of these neurons was influenced by both sound emission and auditory stimuli. Neuronal discharges in response to vocalization, however, differed from those in response to purely auditory stimuli that mimicked the bat call. When an auditory stimulus was temporally locked to a preceding vocalization, the response was dependent on the time delay between the two. This delay-sensitivity completely disappeared when vocalizations were simulated acoustically. Only those vocalization and 'echo' parameters

TABLE 1 Comparison of fossils with two living superfamilies

Character	Fossils	Chthonioidea	Neobisioidea
Cheliceral flagellum	Row of setae	Cluster of setae	Row of setae
Distinct galea	Present	Absent in most	Present in many
Blades of serrula interior	Smooth	Denticulate in adults*	Denticulate in adults*
Number of bothria on palpal fixed finger	6 + 2 †	4 + 2	6
Number of bothria on palpal hand	0	2	0
Teeth of palpal chela	Narrow, inclined	Mostly acute, well-separated	Mostly narrow, inclined
Tarsus of leg 1	Biarticulate‡	Monoarticulate	Biarticulate
Preterminal tarsal seta	Acute	Acute	Modified*

* Condition in early instars not known for certain.

† Count from suspected tritonymph; '6 + 2' refers to six bothria distributed along the finger and two adjacent ones close to the tip.

‡ Observable only on suspected protonymph.

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were encoded that occur in Doppler-shift compensation⁵⁻⁷. In conclusion, I suggest a model for the regulation of the vocalization frequency through auditory feedback.

The horseshoe bat emits long constant-frequency echolocation calls (VOC), which are of a stable individually specific 'resting frequency' when the bat is stationary^{2,3}. The resting frequencies of rufous horseshoe bats (*Rhinolophus rouxi*) are ~78 kHz. The bat's auditory system is tuned to a narrow frequency range ('auditory fovea') of 100–200 Hz above the resting frequency^{1,8}. Flight-induced Doppler-shifts of only a few hundred hertz remove echo frequencies from the auditory fovea. To maintain the Doppler-shifted echo within this foveal range, the horseshoe bat lowers its VOC frequency below the resting frequency by the amount necessary to compensate for the frequency shift in the previous echo³. This Doppler-shift compensation can even be elicited during neurophysiological experiments⁹.

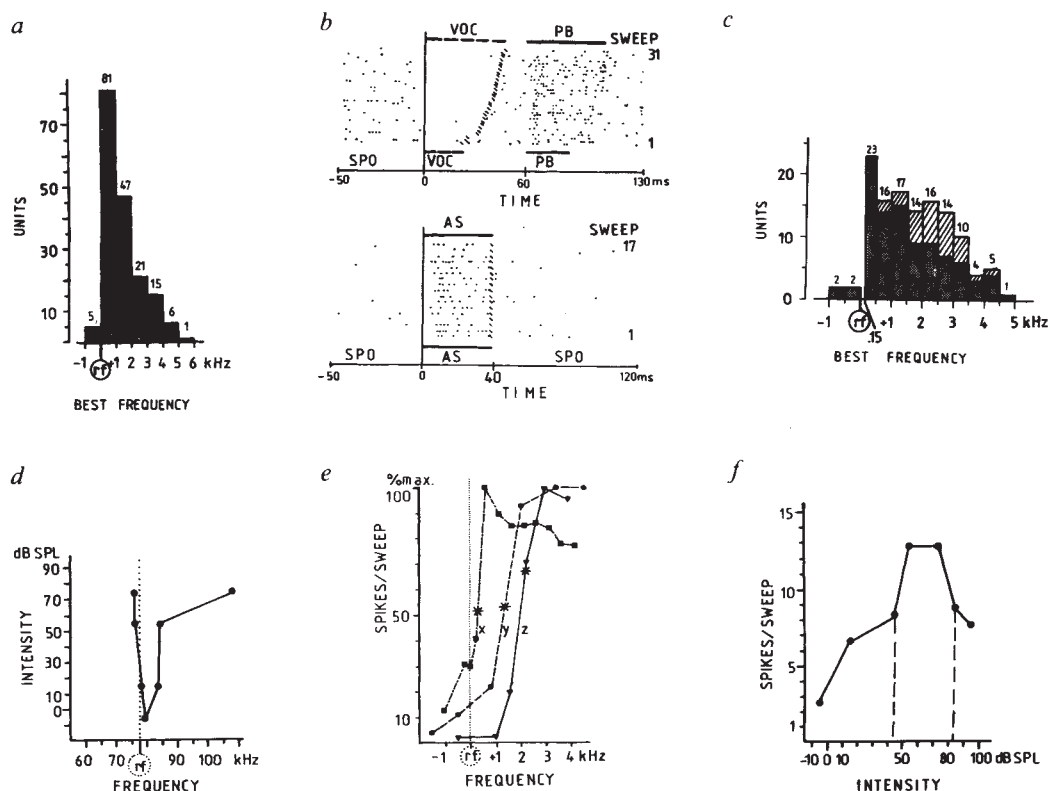
I recorded the activity of single units in the midbrain and brainstem of freely vocalizing rufous horseshoe bats. I chose these brain areas because of their proximity both to auditory structures and to structures implicated in vocalization¹⁰⁻¹³. Of 755 units recorded, 189 units, all located rostral and medial to the nuclei of the lateral lemniscus, were active before, during or after vocalization, and also responded to auditory stimuli. These units were termed 'audio-vocal' neurons (AV neurons)¹⁴. On the basis of its position and connections, the region of these units seems to be homologous to the paralemnisal zone of other mammals¹⁵⁻¹⁸.

All AV neurons were active in the absence of auditory stimuli and vocalization. In 108 units, the background discharge rate increased three- to sevenfold during periods of vocalization. Remarkably, the best frequencies of all but 15 AV neurons were confined to a narrow range between each bat's individual resting frequency and 6 kHz above. This frequency band precisely corresponds to that which evokes Doppler-shift compensation in behaving animals (Fig. 1a)^{3,6}.

Two main classes of AV neurons were found. The first class consisted of 'VOC-inhibitory' neurons (94 of 189 units; Fig. 1b-f) that were suppressed during individual vocalization, whereas auditory stimuli with acoustic parameters identical to the vocalization caused sustained excitation. Most strikingly, these units responded dramatically to frequency changes smaller than 100 Hz near their best frequencies, which were in the range between the resting frequency and 5 kHz above. This sensitivity to frequency variations again matches that of the Doppler-shift compensation^{1,4}. In addition, the non-monotonic spike-count functions typically showed maxima at sound-intensity levels that correspond to naturally perceived echo intensities¹⁹.

'VOC-excitatory' neurons (95 of 189 units) formed the second class of AV neurons. Before the onset of VOC, 63 of them became active. The remaining 32 neurons were only excited synchronously with the onset and end of VOC, or only after the end of VOC. Auditory stimuli identical to vocalization elicited phasic responses in all neurons. Discharges in 38 of the 63 prevocal-excitatory neurons preceded vocalization by up to

FIG. 1 a, AV neurons are tuned to best frequencies between 1 kHz below the resting frequency (RF) and 6 kHz above ($n=179$). b-f, VOC-inhibitory neurons. b, Raster displays of spike occurrence (dots) as function of time (abscissa) over 31 'sweeps' (upper display) and 17 sweeps (lower display) (ordinate). Background activity (SPO) is suppressed during VOC, whereas acoustic VOC-playback (PB) elicits excitation. VOC periods are arranged according to increasing VOC duration from bottom to top. Onsets of VOCs at vertical line, ends of VOCs at slashes. SPO is higher (59 spikes per s) around periods of vocalization (upper display) than during periods of silence (19 spikes per s) (lower display). b (lower display), VOC-identical, artificially generated auditory stimuli (AS) are also excitatory (compare with upper display) (fundamental frequency at RF/2; second harmonic at RF; intensity, 85 dB SPL (dB relative to 2×10^{-5} Pa (sound pressure level)); duration, 40 ms). c, Most neurons had best frequencies from 0.15–5 kHz above RF ($n=94$ dotted columns). Of 29 paralemnisal neurons, 26 failed to show VOC inhibition, but otherwise responded similarly and were tuned between 1 and 4 kHz above RF ($n=29$, hatched columns). No unit was tuned to frequencies between RF and 0.15 kHz above. d, Individual tuning curve. Note shallow slope for frequencies above best frequency. Average $Q_{10\text{dB}}$ -value (best frequency divided by width of tuning curve 10 dB above minimum threshold) near 30. e, Variations of pure-tone frequency of 150 Hz around best frequency (asterisks, three units x, y and z) caused 10% change in discharge rates, which remained high at frequencies 3–4 kHz above best frequencies. f, Spike-count functions are non-monotonic.



METHODS. Single unit activity during vocalization and in response to auditory stimuli were tested in midbrain and brainstem of spontaneously vocalizing rufous horseshoe bats under local anaesthesia. For surgical preparation, stereotactic methods, stimulus presentation, neuronal recording techniques, see refs 22 and 23. Auditory stimuli: playbacks of vocalizations (transient recorder; variable output delay; sample rate, 1 MHz)⁵ and artificially generated auditory stimuli. Harmonic composition of vocalization was retained². Intensities were at acoustically self-perceived VOC level²⁴. Respiration was measured by anemometer. No neuronal activity was linked to expiration alone. Anatomical connections of paralemnisal zone were determined by injection of wheat germ agglutinin to horseradish peroxidase in three bats (TMB and DAB techniques^{15,25,26}). Control injections were in adjacent regions of three additional bats.

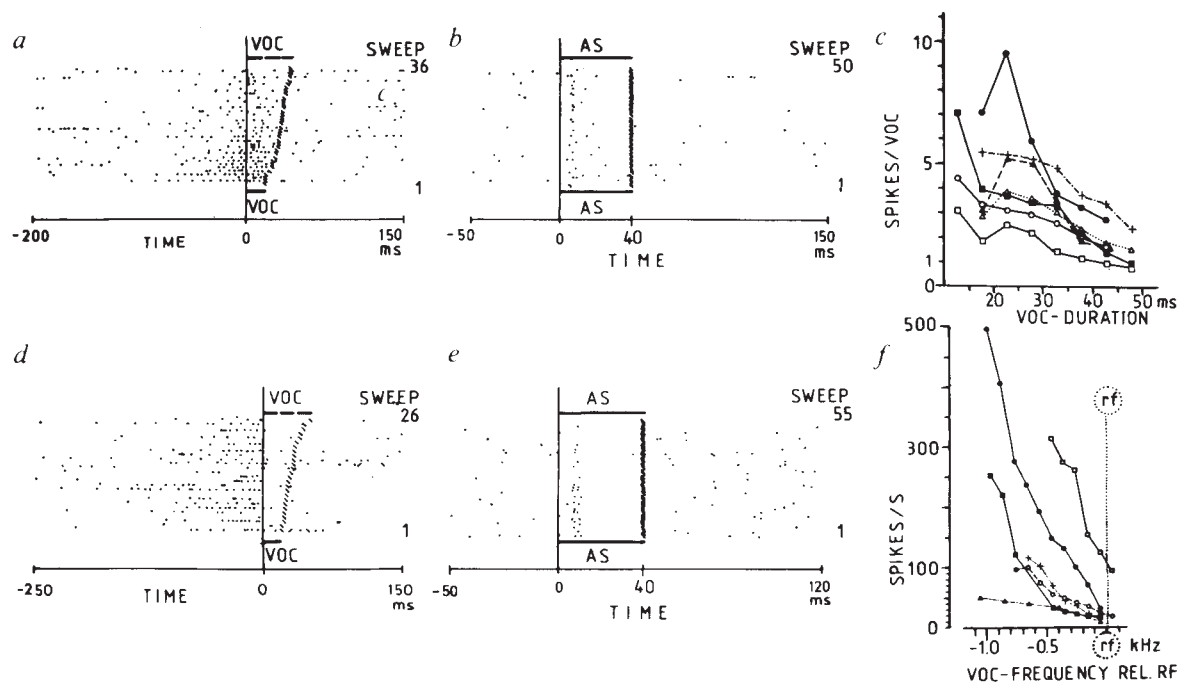
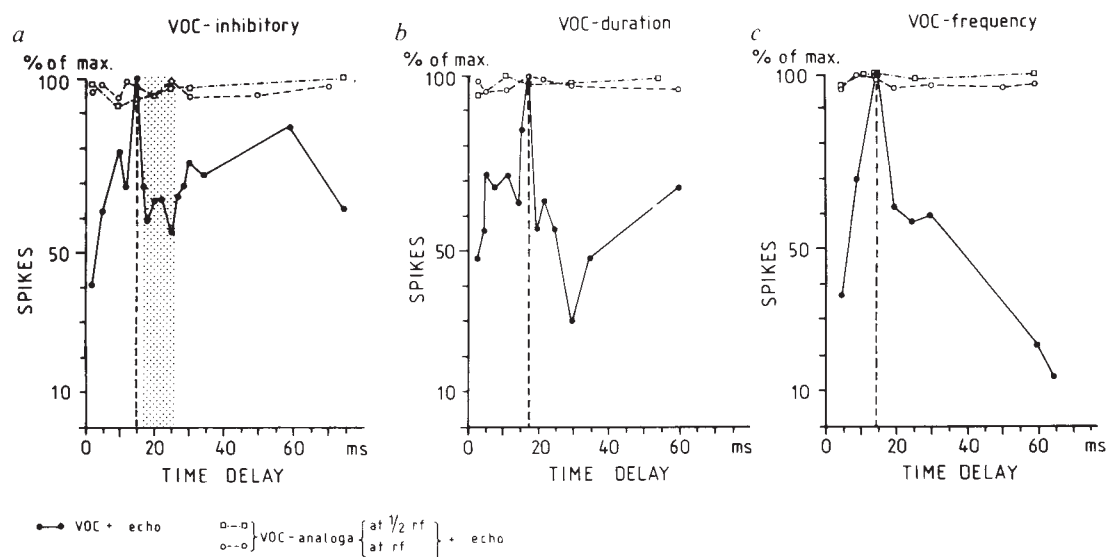


FIG. 2 Pre-vocal excitation was found in 63 of 189 AV neurons. Parts *a, b, d* and *e* follow the convention of Fig. 1*b*. VOC-duration neurons (*a-c*) raised the firing rate as early as 50 ms before the onset of VOC and remained excited during vocalization. Vocalizations are arranged according to increasing VOC length from bottom to top. Note the negative correlation between length of VOC and discharge activity. *b*, Auditory stimulation alone elicited phasic discharges with an average latency of 5.1 ms (± 1.6 ms). *c*, Relationship between the duration of VOC and discharge activity (number of spikes) for seven single units. No correlation with the frequency of vocalization was

found. The discharge rate of VOC-frequency neurons (*d-f*) increased up to 150 ms before the onset of VOC and stopped with the beginning of vocalization. *e*, Auditory stimuli elicited phasic responses with latencies of 10.5 ms (± 2.1 ms) and habituated after the presentation of ~ 40 stimuli. *f*, Frequency and discharge rate are negatively correlated. On average, a decrease of 1 kHz was accompanied by an increase of 75 spikes per s. No correlation with VOC length was observed. VOC frequency relative to resting frequency is plotted against discharge rate.

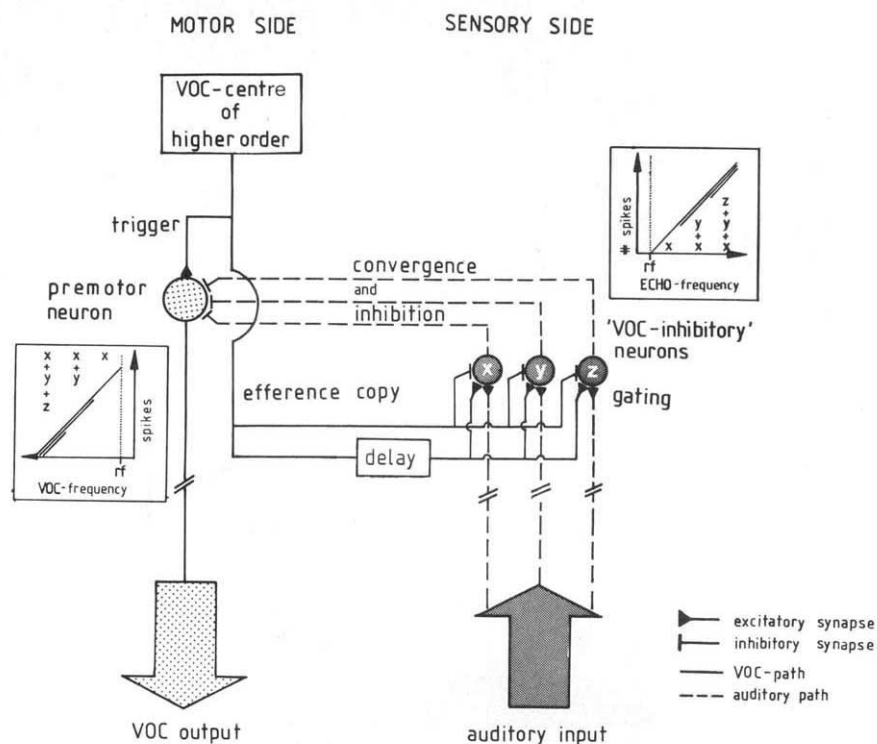
FIG. 3 An auditory stimulus after a vocalization gave a neuronal response to these 'echoes' which was dependent on the intervening time delay. Responses of single unit (relative number of spikes) of each type are normalized with reference to maximal discharge ($\bullet$). Distinct maxima occurred in the VOC-inhibitory (15 units) (*a*) and VOC-frequency neurons (4 units) (*c*) at delays between 5 and 18 ms. In VOC-duration neurons (*b*), 'best' delays varied between 15 and 30 ms (6 units). In all VOC-inhibitory neurons (*a*), auditory responses were reduced up to 50% between 18 and 25 ms (shaded area). This distinct reduction at intervals greater than the optimal delay for excitation was found consistently only in VOC-inhibitory units (*a*). The delay sensitivity completely disappeared in all neurons when vocalization was replaced by an auditory stimulus that simulated the bat's vocalization. Stimuli simulating vocalization at the fundamental frequency, $\square$; VOC-simulating stimuli at second harmonic, $\circ$.



METHODS. After characterizing basic properties of AV neurons, an auditory

stimulus (echo) at the neuron's best frequency, at an intensity of 60–70 dB SPL, and with a duration of 40 ms was triggered by the onset of VOC at a chosen time delay. As a control, vocalization was replaced by an auditory mimic. Harmonic composition of vocalization was retained by using fundamental and second-harmonic frequencies of echolocation call. Intensity of VOC-mimicking signal was 80–90 dB SPL, duration 40 ms. Echo parameters were identical to the VOC-triggered situation.

FIG. 4 Model for the regulation of vocalization frequencies (motor output, left) by VOC-inhibitory neurons coding Doppler-shifted echoes (sensory input, right) (see Fig. 1 *b-f*). During vocalization, the background activity of VOC-inhibitory neurons is suppressed by means of an inhibitory efference copy of the bat's echolocation call (Fig. 1*b*, upper display). Echo information arrives via an excitatory auditory input (Fig. 1*b*, lower display). A delayed excitatory neuronal copy of the vocalization could gate the response to returning echoes of distinct time delays (Fig. 3; ref. 6). Small increments in echo frequency above the resting frequency elicit rises in single-unit activity, until a maximum rate is attained (Fig. 1*e*). Additionally, neurons with higher best frequencies will then be recruited successively (Fig. 1*e*, neurons *x*, *y*, and *z*). Hence, the total activity of this neuronal population will increase with the amount of frequency-shift above the resting frequency (inset on right). If the outputs of VOC-inhibitory neurons converged via an inhibitory synapse (note glutamic acid decarboxylase-positive labelling of paralemniscal neurons) onto pre-motor neurons or motoneurons that show high 'resting' activity at the resting frequency, higher spike rates in this paralemniscal neuronal population *x*, *y* and *z* would lower the spike rates in pre-motor neurons or motoneurons (inset on left), and would thus lower VOC frequencies^{9,21}. The information provided by neurons *x*, *y* and *z* would be stored to affect the subsequent echolocation call triggered some ten milliseconds later^{3,27}.



50 ms (Fig. 2*a-c*). An increase in discharge rates correlated with a decrease in the duration of VOC ('VOC-duration' neurons). Discharges in the remaining 25 prevocal-excitatory units started by as much as 150 ms before the onset of VOC (Fig. 2*d-f*). The discharge rates of these 25 units increased with decreasing VOC frequency ('VOC-frequency' neurons). The encoded call frequencies and lengths match those occurring in Doppler-shift compensation^{3,6}.

In 41 AV neurons, I simulated echo-location situations by triggering an auditory stimulus ('echo') from the onset of VOC and varying the time delay relative to vocalization. Of these, 25 were sensitive to echo delay (Fig. 3). This sensitivity completely disappeared when vocalization was replaced by an identical auditory stimulus. Sensitivity to a delayed echo, therefore, is triggered by the vocalization itself and not by auditory self-stimulation. This demonstrates interactions between auditory processing and vocalization at the single-neuron level. The time delays for the maximum response in VOC-inhibitory and VOC-frequency neurons, ranging from 5–18 ms, correspond to the echo delays necessary for Doppler-shift compensation in horseshoe bats⁶. Additionally, in all VOC-inhibitory neurons, delays ranging between 18 ms and 25 ms resulted in reduced responses to the echo. In rufous horseshoe bats, echoes that return later than 20 ms, fail to induce Doppler-shift compensation (I. Neumann, personal communication; ref. 6).

There are several anatomical connections between the paralemniscal zone and the nucleus ambiguus, the motor nucleus of laryngeal control^{10,15,20}. Most prominent are those via the reticular formation medial to the facial nucleus, and via the extreme rostral part of the inferior colliculus. The physiological properties of AV neurons indicate two mechanisms of VOC frequency control in response to Doppler-shifted echoes.

First, VOC-frequency could be encoded by the firing rate of paralemniscal VOC-frequency neurons, which show increases in spike rates with decreasing VOC-frequencies (Fig. 2*f*). In nucleus ambiguus motoneurons and in the superior laryngeal nerve of the horseshoe bat, however, decreases in VOC-frequencies are caused by decreasing spike rates^{9,21}. The transfer of frequency information from VOC-frequency units to motor com-

mands in the nucleus ambiguus would therefore require inversion by means of inhibition. This hypothesis is supported by the fact that most paralemniscal neurons are positive for glutamic acid decarboxylase, indicating a role for the inhibitory transmitter GABA (γ -aminobutyric acid) in the paralemniscal zone (M. Vater, personal communication). Second, as shown in Fig. 4, VOC-inhibitory neurons could provide sensory information necessary for regulating vocalization frequencies through auditory feedback. Further investigations are required to determine whether the transformation of this sensory information to final motor commands takes place at the level of the paralemniscal zone.

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Optical imaging of calcium accumulation in hippocampal pyramidal cells during synaptic activation

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THE dynamic response of nerve cells to synaptic activation and the spatial distribution of biochemical processes regulated by ion concentration are critically dependent on the cell-surface distribution of ion channels. In the hippocampus, intracellular calcium-ion concentration is thought to influence the biochemical events associated with kindling^{1,2}, excitotoxicity^{3,4}, and long-term potentiation⁵⁻¹². Computer models of hippocampal pyramidal cells¹³ also indicate that calcium-channel location influences dynamic characteristics such as bursting^{14,15}. Here, we have used *in situ* microfluorometric imaging¹⁶ in brain slices to directly measure the spatial distribution of calcium accumulation in guinea-pig CA1

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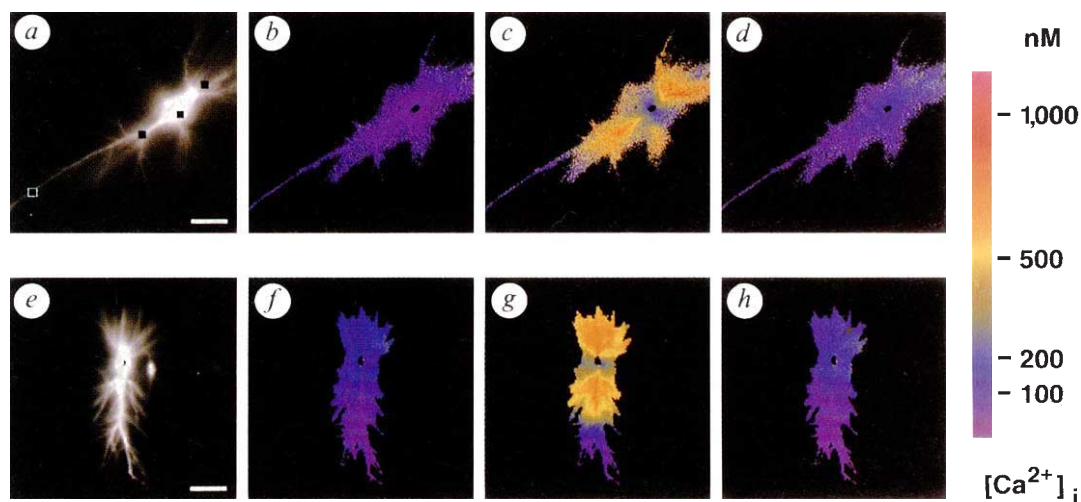
FIG. 1 a, Fluorescence image of a CA1 pyramidal cell filled with fura-2 by microinjection (380 nm excitation; 300 × 300 pixels). The fluorescence intensity was used to create a mask that defined where ratios and the associated $[Ca^{2+}]_i$ were subsequently computed. **b-d**, Maps of $[Ca^{2+}]_i$ before (b), during (c), and 17 s after (d) orthodromic synaptic activation of the cell in a produced by stimulation of the stratum radiatum (50 Hz, 2 s). **e**, Fluorescence image (380 nm excitation; 150 × 150 pixels) of a different CA1 pyramidal cell. **f-h**, Maps of $[Ca^{2+}]_i$ before (f), during (g), and 50 s after (h) orthodromic synaptic activation (20 Hz, 10 s) of the cell shown in e. Dark pixels within the soma represent locations where fluorescence saturated the CCD (charge-coupled device) camera; ratios are therefore not displayed. Scale bars, 100 μ m.

METHODS. Experiments were performed on 300- μ m thick transverse slices of hippocampus from 90-400-g guinea pigs. Slice preparation and maintenance *in vitro* followed standard procedures^{25,26}. For imaging, slices were placed on a glass coverslip that was the bottom of a submerged slice chamber, and continuously perfused with saline (30-32 °C) of the following composition (mM): NaCl, 124; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.6; $NaHCO_3$, 26; glucose, 10; equilibrated with 95% O_2 /5% CO_2 . CA1 pyramidal neurons were injected with microelectrodes pulled on a Brown-Flaming puller (Sutter) from 1.5-mm tubing (No. 6030 AM Systems) containing a 12-mM solution of the K^+ salt of fura-2 (ref. 17) (Molecular Probes) in 100 mM KCl. After beveling (Model BV-10, Sutter), electrode resistance was 150-200 M Ω . Cells having a stable resting potential of ≥ -50 mV and responding to brief depolarizations with a burst of action potentials were injected with fura-2 by continuous iontophoretic current (-1 nA; 10 min). Only neurons located within 100 μ m of the surface of the slice were used in these experiments; at greater depths image clarity was reduced. Orthodromic activation of CA1 neurons was produced by stimulation of the stratum radiatum (300-600 μ m from the cell, midway between stratum moleculare and stratum pyramidale) with bipolar tungsten electrodes (25 μ m diameter, polyimide coated; California Fine Wire) with trains of 400-1,000 μ A current pulses (100 μ s pulse

pyramidal cells during trains of orthodromic synaptic stimulation. Calcium accumulation is substantial throughout the entire proximal section of the apical and basal dendrites. Most of this accumulation results from influx through non-NMDA (N-methyl-D-aspartate) voltage-gated calcium channels, and in the apical dendrite it drops steeply as the dendrite enters stratum moleculare, the termination zone of perforant path afferents. These results demonstrate a marked segregation of calcium-channel activity and directly show a spatial distribution of calcium accumulation during orthodromic synaptic activation.

Individual pyramidal cells in the CA1 region of a guinea-pig hippocampal slice were filled with the pentapotassium salt of fura-2 by iontophoretic microinjection. Maps of intracellular free-calcium concentrations ($[Ca^{2+}]_i$) were determined from the ratio of low-magnification fluorescence images (Fig. 1a, e) excited at 340 nm and 380 nm (ref. 17). Exposure times were 0.4-2 s and the calculated concentrations represent time-averaged values of $[Ca^{2+}]_i$.

Orthodromic synaptic activation of pyramidal cells through stimulation of the stratum radiatum with bipolar electrodes produced widespread calcium accumulation and large spatial gradients in the dendrites. The stimulus conditions used were based on those widely employed in long-term potentiation (LTP) experiments and reliably produced LTP in control slices (see Fig. 1 legend). As shown in the colour-coded $[Ca^{2+}]_i$ maps in Fig. 1b-d, stimulation at 50 Hz for 2 s produced a large increase in $[Ca^{2+}]_i$ in the proximal-apical and basal dendrites (Fig. 1c),



duration). In control slices using 0.1 Hz test pulses, the evoked field potentials (measured using extracellular electrodes, No. 6030 AM Systems, 2 M NaCl, 4-6 M Ω) were 0.3-0.8 mV in stratum radiatum and the population spike in stratum pyramidale 0.5-3.5 mV. Tetanic stimuli (50 Hz, 2 s) and (20 Hz, 10 s) reliably induced potentiation in 10 control slices, as indicated by an increase in the slope of the field potential in stratum radiatum (average 25% increase, range 18%-33%, $n=4$) and an increase in the amplitude of the population spike in stratum pyramidale (average 216%; range 139%-343%; $n=6$). Cells were viewed from the top surface of the slice using an upright microscope (Zeiss UEM) and a Nikon $\times 10$ UVF objective covered with Saran Wrap (Dow) for water-immersion use. Alternatively, cells on the bottom of the slice were viewed through the glass coverslip using an inverted microscope (Zeiss IM35) with the same objective but without the Saran Wrap. Images were taken with cooled CCD cameras (model 220, Photometrics) with Macintosh II (Apple) controlled image acquisition and display. $[Ca^{2+}]_i$ was determined from images at 340 nm and 380 nm excitation using the ratio method^{17,27}. Reported $[Ca^{2+}]_i$ values assumed a viscosity correction corresponding to a 30% reduction in the 340/380 nm fluorescence ratio²⁸. Although several factors can contribute to errors in our calculations of absolute concentrations (non-uniformity of background fluorescence, choice of viscosity correction factor), under static conditions computed fluorescence ratios varied by less than 5%. In Figs 2-4, times refer to the midpoint of image pairs relative to the start of stimulation.

which recovered to the pre-stimulus level following stimulation (Fig. 1d). Figure 1f-h shows a similar sequence of $[Ca^{2+}]_i$ maps for a different pyramidal neuron (20 Hz, 10 s stimulation). This same basic pattern of calcium accumulation with afferent activation was measured in all 26 pyramidal cells tested.

The time course of calcium accumulation and recovery for the experiment shown in Fig. 1b-d is plotted in Fig. 2 for the four locations indicated in Fig. 1a: basal dendrite, soma, proximal apical dendrite and distal apical dendrite. During the 2 s stimulation period, the largest (more than sevenfold in this cell) and most rapid accumulation occurred in the proximal dendrites; accumulation in the soma lagged behind the dendritic response and was greatly reduced in amplitude. Only a small increase was observed in the distal apical dendrite. For the 26 cells measured, stimulation at 50 Hz for 2 s, or 20 Hz for 5 or 10 s, produced peak calcium levels in the basal and apical dendrites, that ranged from 600 to 2,000 nM; calcium accumulation in these regions decreased by 50% in 1-5 s after stimulation. The return to pre-stimulus levels for the soma was somewhat slower, requiring 10-20 s to decrease by 50%. All regions eventually returned to the pre-stimulus levels, requiring 30-90 s for the dendrites and ~5 min for the soma.

To demonstrate that the calcium accumulations were produced by synaptic activation and to rule out direct depolarization

of the pyramidal cell by the stimulating electrode, postsynaptic currents were blocked with the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (ref. 18) (CNQX; 40-100 μ M; Tocris Neuramin). Figure 2b shows plots of calcium accumulations with time in representative sections of apical and basal dendrites and soma in normal saline (solid curves) and after bath application of CNQX (40 μ M; dashed curves). The tetanus-induced calcium accumulation was completely blocked by CNQX ($n=5$). In separate experiments, afferent-fibre activation was blocked by bath application of the voltage-dependent sodium-channel blocker tetrodotoxin (TTX; 1 μ M; Sigma). Tetanus-induced calcium accumulation was also completely blocked under these conditions ($n=3$; data not shown). The calcium accumulations observed in normal saline were produced, therefore, by synaptic activation of the pyramidal cell.

Quantitative comparison of calcium distribution under different conditions was facilitated by plotting $[Ca^{2+}]_i$ against distance along the dendrite. In Fig. 3b the average levels of $[Ca^{2+}]_i$ in small regions (Fig. 3a) along the lengths of apical and basal dendrites are plotted (solid lines) against distance from the cell-soma centre for the primary apical dendrite and a representative basal dendrite. Individual curves for four time points during the rising phase of calcium accumulation are superimposed. In the 26 cells analysed in this manner, the peak accumulation level along the apical dendrite occurred at an average distance of $105 \pm 30 \mu$ m from the soma, with a sharp decline in accumulation between 150 and 250 μ m. The gradient of free calcium along this declining section was typically 5-20 nM per μ m at the peak of the accumulation. The half-maximal distance from the cell soma was $180 \pm 50 \mu$ m. Very little accumulation was observed in distal dendritic regions corresponding to stratum moleculare (a mean increase of 40 ± 30 nM at 400 μ m from the soma, $n=16$), which in this preparation begins 250-380 μ m from the cell-body layer.

In all cells tested ($n=26$) apical dendritic activation by stimulation of the stratum radiatum also produced increases in the basal dendrites. Similarly in all of five cells tested, basal dendritic activation by stimulation of stratum oriens produced both basal and apical dendritic accumulations. These results indicate that most of the measured accumulation was produced by action-potential invasion or spreading depolarization which activated voltage-dependent calcium conductances. To distinguish between transmitter-gated calcium fluxes produced by NMDA-receptor activation and those due to activation of strictly voltage-dependent calcium channels¹⁹, concentrations produced by action-potential trains driven by current pulses from an intracellular microelectrode were measured. The effect of NMDA-receptor blockers on the calcium accumulation was also determined.

During intracellular microelectrode impalements (Fig. 3c), cell-soma images were corrupted by the dye-containing microelectrode, but measurements could still be made reliably in dendritic areas and other unobstructed parts of the soma. Calcium accumulation for representative apical and basal dendrites is plotted in Fig. 3d for both a quiescent period and during action-potential stimulation by depolarizing current pulses (20 Hz). In all of seven neurons stimulated in this manner, large increases in the proximal apical and basal dendrites were observed with a strong decay of accumulation in the apical dendrite at soma distances of 150-250 μ m. Peak calcium accumulation in the principal apical dendrite occurred between 65 and 210 μ m from the soma. These intracellular microelectrode experiments provide further evidence that the accumulations observed with orthodromic synaptic stimulation were produced primarily by non-synaptic voltage-dependent calcium fluxes.

Under the present experimental conditions, bath perfusion with the NMDA-receptor antagonist DL-2-amino-5-phosphonovaleric acid (AP5; 100-200 μ M; Sigma) produced no statistically significant change in the levels of calcium accumula-

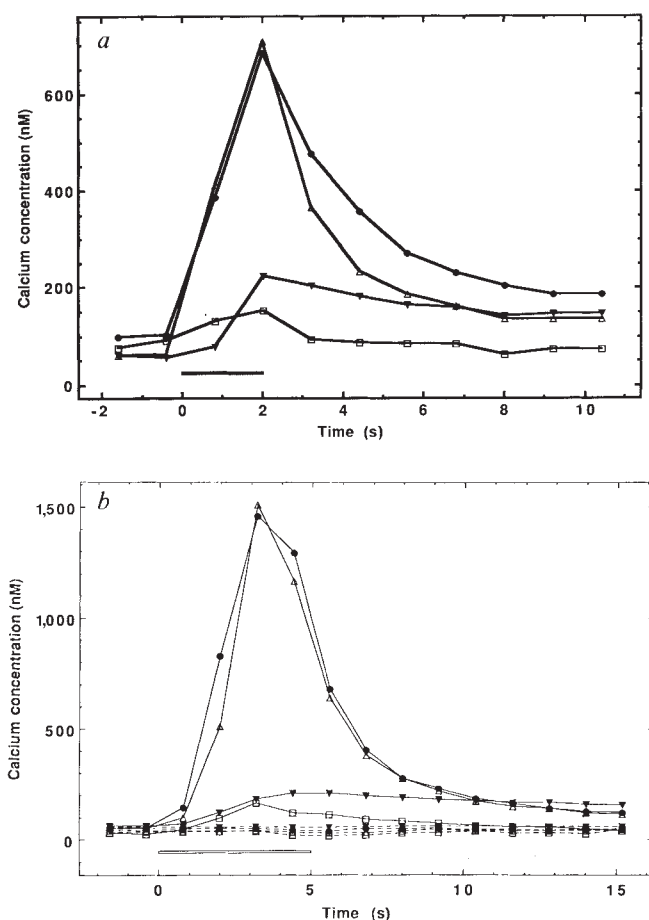


FIG. 2 a, Graph of calcium accumulations against time at the four spatial positions indicated in Fig. 1a. Circle, basal dendrite; triangle, proximal apical dendrite; inverted triangle, soma; square, distal apical dendrite. During the period indicated, the pyramidal cell was stimulated (50 Hz, 2 s) with current pulses applied to a bipolar electrode located in the stratum radiatum. b, Effect of CNQX on calcium accumulations produced by stimulation (20 Hz, 5 s) for a different pyramidal cell. Plot key as in a. Solid lines show accumulations in normal saline against time. Dashed lines show complete block of the response following bath application of 40 μ M CNQX.

tion produced by orthodromic synaptic activation (Fig. 4). Cells were first bathed in AP5 and images taken during tetanic stimulation (20 Hz; 10 s); after extensive washout (15–30 min) a second set of images at identical time points was taken during identical stimulation. In eight experiments comparing the stimulation of stratum radiatum in AP5-containing medium with that in normal medium, the average reduction of apical dendritic calcium accumulation was $3 \pm 10\%$, compared with an average reduction of $2 \pm 10\%$ for basal dendrites (Fig. 4). Similar results were obtained for side branches of the primary apical dendrite. Three cells showed decreases in the peak calcium levels reached in the proximal apical dendrite, but not basal dendrites, in AP5-bathed cells compared with the control condition (Fig. 3*b*; dashed lines). Under our experimental conditions, a contribution to calcium accumulation caused by influx through NMDA-receptor channels during tetanic afferent stimulation was difficult to resolve because of variability between trials and the presence of much larger AP5-insensitive accumulations.

These *in situ* determinations of calcium changes in hippocampal pyramidal cell dendrites responding to presynaptic stimulation are a significant advance. They demonstrate that intracel-

lular depolarization and tetanic orthodromic stimulation of the Schaffer collateral afferents to CA1 hippocampal pyramidal cells cause a large transient accumulation of $[Ca^{2+}]_i$ throughout the proximal apical and basal dendrites. At these locations, the second messenger functions of calcium might be employed to activate calcium-dependent biochemical processes. In our experiments, synaptic activation did not produce the sustained dendritic calcium gradients that have previously been measured in accurately dissociated hippocampal neurons in response to NMDA (ref. 20). The accumulation during stimulation seems to be produced primarily by the activation of voltage-dependent calcium channels localized on the proximal apical and basal dendrites, although a contribution to calcium accumulation by release from internal stores cannot be ruled out. The accumulation in proximal apical dendrites is consistent with electrophysiological evidence for voltage-dependent calcium conductances in CA1 hippocampal pyramidal cell dendrites^{21–23}. We have also shown calcium accumulation in the basal dendrites and a strong decay of accumulation along the apical dendrite. In distal dendritic regions beyond 250 μm from the soma, little accumulation is observed. In the guinea pig hippocampus, the stratum

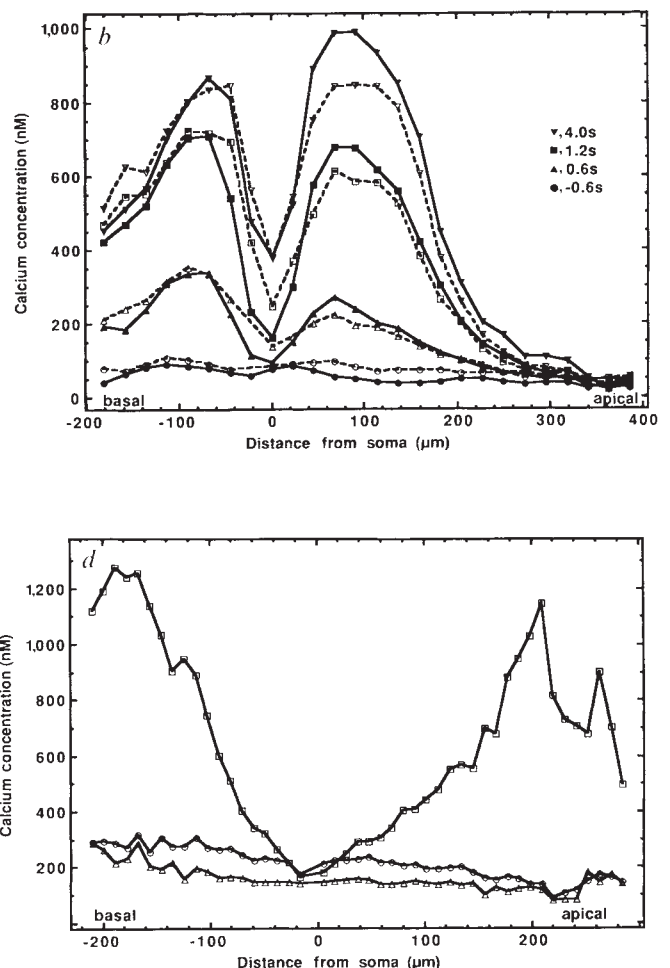
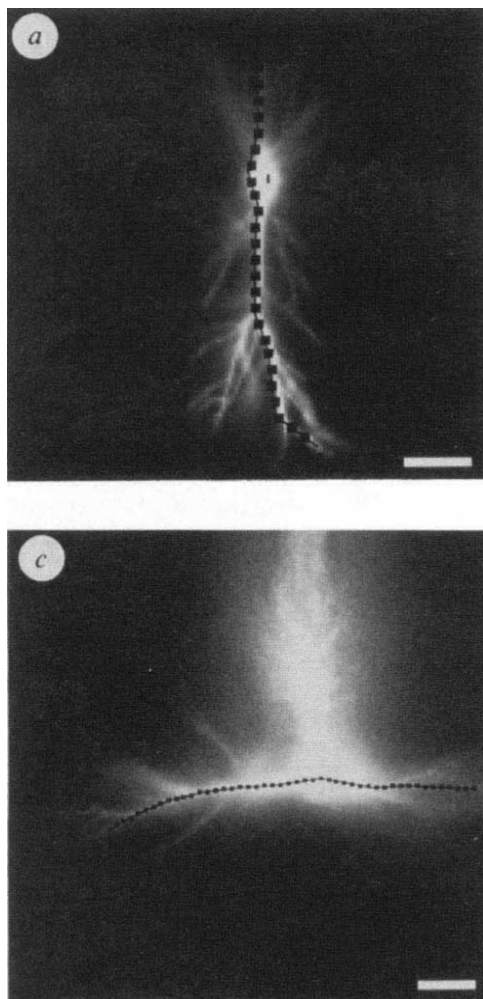


FIG. 3 *a*, Fluorescence image (380 nm excitation) of the cell shown in Fig. 1*e* with superimposed string of analysis boxes traversing sections of apical and basal dendrite. Calcium accumulations were calculated within each box for image pairs taken during orthodromic activation of Schaffer collateral afferents (20 Hz, 10 s). *b*, Graph of calcium accumulation in the analysis boxes shown in *a* against distance from the cell soma centre in the presence of AP5 (dashed lines) and after washout (solid lines). Curves are shown for several time points during the increasing accumulation phase of the

response. *c*, Fluorescence image (380 nm excitation) with superimposed analysis boxes of CA1 pyramidal cell penetrated by a fura-2 filled intracellular microelectrode used to fill the cell with indicator and to inject depolarizing current. *d*, Graph of calcium levels measured in the boxes shown in *c* against distance from the cell soma for image pairs taken at rest and during a train of depolarizing current pulses (20 Hz). Triangle, before; circle, after; square, during stimulation. Scale bars, 100 μm .

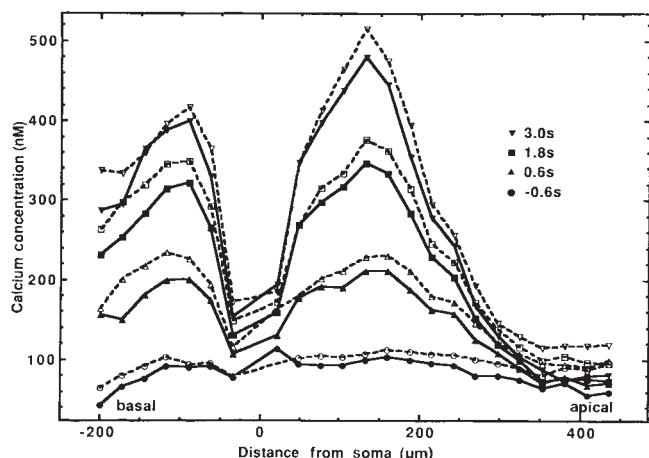


FIG. 4 Graph of calcium accumulation in dendritic regions of a pyramidal cell (image not shown) against distance from the cell soma centre in the presence of AP5 (dashed lines) and after washout (solid lines). Curves are shown for several time points during the increasing accumulation phase of the response.

molecular region begins 250–380 μm from the cell soma layer and contains both perforant path inputs from entorhinal cortex and thalamic afferents that are distinct from the Schaffer collateral system (CA3 efferents) that synapse in the stratum radiatum and stratum oriens. The sharp calcium gradient we observed probably reflects a lower density of active voltage-gated calcium channels in the distal dendrite, as the electrotonic properties of CA1 cells are not consistent with a sharp voltage gradient²⁴. We surmise that this calcium channel localization represents the physical substrate for a different computational or learning role of proximal and distal apical dendritic segments. *Note added in proof:* In recent experiments similar to those shown in Fig. 3b, but employing higher stimulus frequencies and increased temporal resolution, we have reliably resolved a transient component of calcium accumulation that is blocked by AP5 and localized to dendritic regions near activated afferent fibres. □

Release of endogenous excitatory amino acids from turtle photoreceptors

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RESPONSES to light are transmitted from photoreceptors to second-order retinal neurons by chemical synapses that may use an excitatory amino acid (EAA) as the neurotransmitter. This hypothesis is based primarily on the pharmacological actions of EAA agonists and antagonists on the membrane potentials and light responses of second-order neurons^{1,2}. But the release of endogenous EAAs, which is a critical criterion for the identification of EAAs as transmitters³, has not been demonstrated. Here we report the use of outside-out membrane patches excised from rat hippocampal neurons to detect the release of EAAs from synaptic terminals of isolated turtle photoreceptors. Electrical stimulation of or application of lanthanum chloride to photoreceptors induced an increase in the frequency of opening of 50-pS channels in the patches. These channels were identified as the class of glutamate-activated channels that are also gated by aspartate and NMDA (*N*-methyl-D-aspartate). In several photoreceptor–patch pairs, spontaneous channel activity was observed near the synaptic terminals. These results provide strong evidence to support the hypothesis that both rods and cones of the turtle use an EAA as their neurotransmitter.

Excised membrane patches that contain ion channels gated by nicotinic cholinergic agonists have been used to sense the release of acetylcholine from growth cones of developing neurons^{4,5}. We used EAA-sensitive membrane patches to test whether there is a detectable release of a glutamate-receptor agonist from the synaptic terminals of individual rods and cones. Outside-out patches were excised from rat hippocampal neurons that had been grown in primary culture⁶ (see Fig. 1 legend). Both EAA-activated and non-EAA-activated channels are found in these patches. Our strategy was to create a sensitive and selective probe for EAAs by pharmacologically blocking all non-EAA-gated channels (see Fig. 1 legend). Under these conditions, we characterized the properties of patches taken from the same or similar batches of hippocampal cells as those that were used to detect release. Every patch responded to exogenous L-glutamate, L-aspartate and NMDA with channel openings identified as NMDA-gated channels. The channels exhibited the typical 45–50-pS conductance (and subconductance states^{6–8}), were blocked by the specific antagonist D-2-amino-5-phosphonovalerate⁶, had reversal potentials close to 0 mV, were blocked by elevated Mg^{2+} at hyperpolarized potentials⁷ and were sensitized by glycine⁹. In test solutions containing 2 μM glycine, an increase in NMDA-gated channel activity was readily discernible at NMDA concentrations of 0.5 μM and glutamate concentrations of 0.1 μM .

Acutely isolated photoreceptors from dissociated turtle retinas¹⁰ (see Fig. 1 legend) and cultured hippocampal cells were placed in the same media-filled recording chamber. An outside-out patch was detached from a hippocampal neuron, positioned within 2 μm of a photoreceptor synaptic terminal, and a stimulating electrode was placed so that it just touched the distal end of the inner segment of the photoreceptor. Figure 1c shows the electrodes positioned near an isolated rod photoreceptor. At least one control run of channel activity was always recorded before stimulation. Photoreceptors were stimulated with pulses of positive voltage (0.1–5 V for 50–1,000 ms) applied through the stimulating electrode. Such stimuli were expected

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to depolarize the synaptic terminals in the same manner as the trans-retinal currents that have been passed across intact retinas to evoke transmitter release from photoreceptors¹¹. Figure 1(a, b) displays sample records of channel openings (downward deflections) recorded from an outside-out patch positioned near the synaptic terminal of a yellow cone during a control period and immediately after a 500-ms stimulus pulse. Within 200 ms after the onset of stimulation, an increase in channel activity was observed. After an interval of increased channel activity lasting up to 30 s, the probability of channel opening returned to pre-stimulus levels (see Fig. 1d).

Channel activity was quantified by integrating the total current that flowed across the patch during each trial period. Figure 1d shows that the time-averaged current induced by electrical stimulation (trial 3) was ~10 times higher than the time-averaged current for the previous two control runs. In 5 of 13 photoreceptor-patch pairs, the increase in the time-averaged current due to electrical stimulation was more than nine times greater than the control recordings (mean increase, 16.8-fold; range 9.6–31.1-fold; two yellow cones, two red cones and one rod). In two of these pairs, subsequent electrical stimulation after a delay of 60–120 s elicited a second, but smaller, increase in channel activity (5.4- and 9.9-fold increase in time-averaged current). These results suggest that most of the releasable neurotransmitter was secreted as a result of the initial stimulus. In the remaining pairs there was no discernible change in channel activity upon stimulation. Several possibilities could account for these failures: some photoreceptors could have been metabolically compromised during the dissociation and unable to release transmitter; the patches may have contained few channels and therefore had low sensitivity; the synaptic terminals

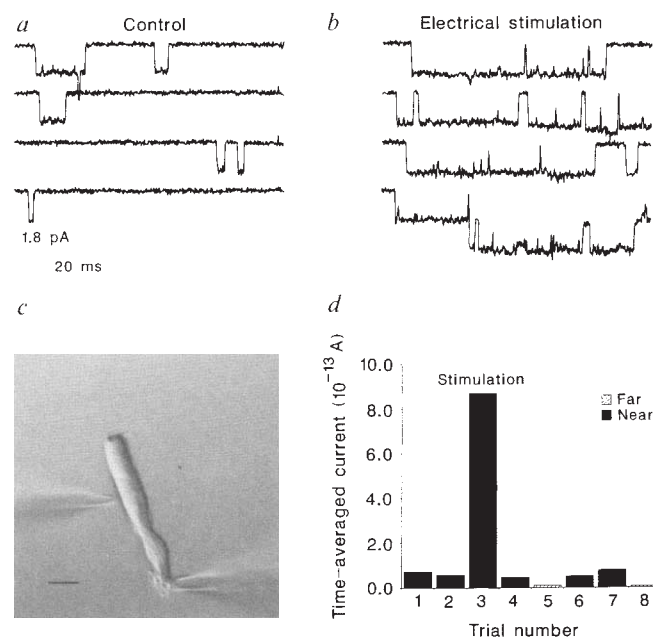
may have been inadequately depolarized because of the low sealing resistance of the stimulating electrode; or the patch could have been too far from a release site for detection. Meriney *et al.*¹² have shown that acetylcholine-sensitive patches are unable to detect acetylcholine release when positioned more than 2–3 μm from a release site.

Spontaneous channel openings, presumably reflecting the release of EAAs from the aggregate retinal neurons, were usually observed regardless of the position of the membrane patch in the recording chamber. The amount of spontaneous channel activity that could be recorded near the photoreceptor terminal, however, was at least four times that recorded at positions 50 μm away (Fig. 1d). These findings held for five out of eight photoreceptor-patch pairs (mean increase, 12.54-fold; range 4.3–40.0-fold; one red cone, one blue cone, two yellow cones, two rods). Although the normally short lifetime of the membrane patches made it difficult to quantify significant differences between low rates of channel activity, we found no difference in the rate of channel activity measured just adjacent to the inner segment compared with that measured 50 μm away.

Potassium and lanthanum were applied to photoreceptors to evoke transmitter release. High K^+ (50 mM) applied directly to outside-out patches, however, usually resulted in flickering inward-going channel events, which probably represented K^+ conductances. These events made quantification of NMDA-channel activity impossible. Lanthanum chloride has been used as a secretagogue at the neuromuscular junction¹³ and in the retina¹¹. Lanthanum chloride was applied from pressure pipettes to the inner segments of isolated photoreceptors. In three of seven photoreceptors (two yellow cones, one rod) the application of La^{3+} resulted in a 3.3-, 12.3- and a 3.6-fold increase in

FIG. 1 Stimulation of isolated photoreceptors induces NMDA-channel activity in outside-out patches positioned adjacent to synaptic terminals. *a*, Selected channel openings recorded with the patch positioned within 2 μm of the synaptic terminal of a yellow cone immediately before photoreceptor stimulation. *b*, Records obtained just after extracellular electrical stimulation of the cone. Patch holding potential, -40 mV . Channel openings are downward (inward current). *c*, Isolated turtle rod with patch pipette positioned near the synaptic terminal and stimulating pipette positioned near the inner segment. Scale bar, 10 μm . *d*, Effects of stimulation of the same cone as in *a* and *b*, and position of the patch pipette on channel activity. In each trial, the current was integrated and normalized by dividing by each trial duration, which ranged from 3.28 to 65.12 s (owing to the sampling routine that stored only those 1,024-point sample epochs that contained at least one opening). In trials 1–4 and 6–7, the NMDA-channel membrane patch was positioned near the base of the synaptic terminal of the yellow cone. In trial 3 (corresponding to the records in *b*), current was passed from the stimulating electrode to depolarize the membrane of the cone terminal. In trials 5 and 8 the patch pipette was located $\sim 50\text{ }\mu\text{m}$ away from the terminal but still at the same height ($\sim 1\text{--}3\text{ }\mu\text{m}$) from the bottom of the dish to measure background channel activity gated by the bath perfusate (no photoreceptor stimulation).

METHODS. Neurons were obtained from the CA1 region of the hippocampus from 1–3-day-old Long-Evans rats. Dissociation and cell culture methods are described previously⁶. Photoreceptors were dissociated from isolated retinas dissected from *Pseudemys scripta* turtle eyes. Strips (1 mm wide) were incubated in a low Ca^{2+} (no added Ca^{2+} , 10 mM EDTA), cysteine-enriched (1–2 mM) papain solution (7 U ml^{-1} , Worthington) for 30 min, rinsed in Ringer's solution and then triturated in a BSA-coated pipette. Aliquots of supernatant that contained isolated cells were dropped onto glass coverslips covered with collagen/concanavalin A (ref. 10) and allowed to attach for at least 10 min before recording. The external recording solution contained (in mM): NaCl, 165; KCl, 3; CaCl₂, 2–4; picrotoxin, 0.05; strychnine, 0.001; tetrodotoxin, 0.0005; glycine, 0.002; HEPES buffer, 5 (pH adjusted to 7.4 with NaOH). The internal solution in the patch pipette contained (mM): Cs gluconate, 140; CsCl or NaCl, 10; EGTA, 10; HEPES buffer, 5 (pH adjusted to 7.4 with CsOH). These ionic substituents, buffers and antagonists were chosen to suppress voltage- and ligand-gated channel events commonly found in outside-out patches excised from hippocampal neurons to make selective EAA probes. The internal solution for whole-cell recordings from photoreceptors also contained 1–2 mM CsF. The capacity of the recording



chamber was $\sim 0.4\text{ ml}$. The superfusion rate was $\sim 1.5\text{ ml min}^{-1}$. Extracellular stimulating electrodes were lightly fire-polished patch pipettes filled with external solution. Experiments were performed at room temperature ($21\text{--}25^\circ\text{C}$) on the stage of a Zeiss IM35 inverted microscope using $\times 40$ water-immersion or $\times 63$ oil-immersion objectives and DIC optics. Lanthanum chloride at an electrode concentration of 5 mM was applied by pressure from unpolished patch pipettes positioned within 10 μm of the photoreceptor inner segment. NMDA, L-glutamate, L-aspartate and acetylcholine were applied to outside-out patches from perfusion tubes with inner diameters of $\sim 200\text{ }\mu\text{m}$. Solution changes were effected by either moving the perfusion tubes or the patch pipettes, and were completed within 10 ms as determined by the current relaxation that was induced by switching to iso-osmolar KCl-based saline from the normal NaCl-based external solution.

channel activity recorded near the synaptic terminal. Figure 2a illustrates the channel activity evoked by ejection of La^{3+} on a yellow cone. Figure 2b shows the time-averaged current for three control trials followed by three applications of La^{3+} to this same cone. It is clear from this figure that the second and third applications did not induce the same large increase in channel activity and, in fact, may have slightly decreased channel activity.

To test the possibility that La^{3+} might have been directly activating channels in the patch, we compared channel activity in 5 μM NMDA and 1 μM glycine (upper two traces, Fig. 3a) with that recorded in the presence of agonist in addition to 5 mM La^{3+} (middle traces, Fig. 3a). We found that La^{3+} did not induce channel activity in the patches but instead reversibly blocked NMDA-activated channels in a voltage-independent manner similar to the effect of zinc¹⁴. Thus, the increase in channel activation observed with La^{3+} was due to the action of La^{3+} on the photoreceptors. The lack of an increase in channel activity with subsequent applications of La^{3+} may have been due to the delayed block of the NMDA-gated channels by La^{3+} as it diffused to the patch pipette from the region of application or to the depletion of synaptic transmitter in the terminals, or both. In support of the depletion possibility, preliminary electron microscopy of photoreceptor synaptic terminals revealed an almost complete depletion of synaptic vesicles in La^{3+} -treated photoreceptors.

Depolarizing the photoreceptor membrane potential in the whole-cell recording mode proved to be ineffective in evoking transmitter release. With NMDA-sensitive patches near the synaptic terminals, the membrane potential of the isolated rod or cone was stepped from a holding potential of -60 mV , at which level transmitter release is fully suppressed^{15,16}, to 0 mV ,

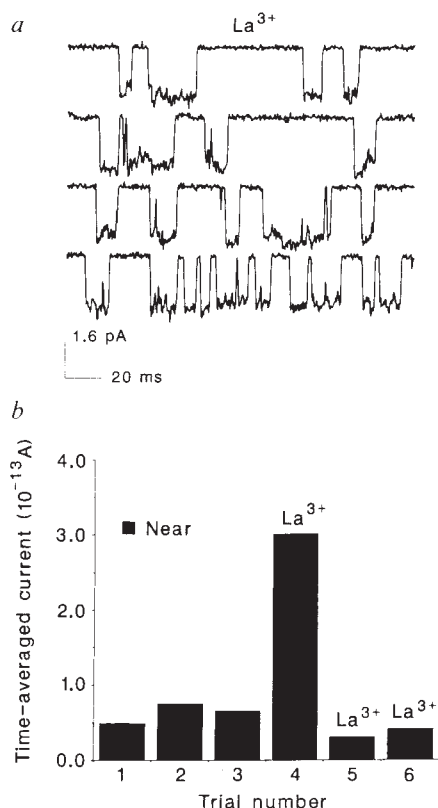


FIG. 2 Channel activity induced by La^{3+} perfusion of the photoreceptor. *a*, Sample records of NMDA channel activity immediately after pressure injection of La^{3+} onto the inner segment of an isolated rod. *b*, Time-averaged current measured before and during three separate pressure ejections of a 1-s pulse of La^{3+} on to the same rod as shown in *a*. The membrane patch was located near the synaptic terminal throughout the course of this experiment. Holding potential, -40 mV .

at which level release should be maximal. No change in channel activity in the outside-out patch was observed in all of nine photoreceptor-patch pairs. To test whether dialysis of the interior of the cell during the whole-cell recordings could have disrupted the release process, the pipette potential was held at 0 mV during the transition from the on-cell to the whole-cell configuration. This effectively stepped the photoreceptor membrane potential from rest to 0 mV on breaking into the cell. For two of the three cases tested in this manner, increases in channel activity in the outside-out patch were observed within 200 ms of forming the whole-cell recording from the photoreceptor (Fig. 3b). These results indicate that, at least transiently, transmitter release mechanisms were intact in photoreceptors and that the whole-cell recordings may have disrupted these mechanisms.

We have measured evoked and spontaneous channel activity in NMDA-sensitive outside-out patches near the basal surface of the synaptic terminals of photoreceptors. These channels were identified as EAA-gated NMDA channels. No channel activity was induced by GABA (γ -aminobutyric acid) or acetylcholine, two putative photoreceptor neurotransmitters¹⁷⁻¹⁹. GABA-gated channels were blocked with picrotoxin and would have produced outward-going currents at -40 mV with the chloride concentrations used in our experiments. Neither the patches nor the hippocampal neurons, recorded in whole-cell mode,

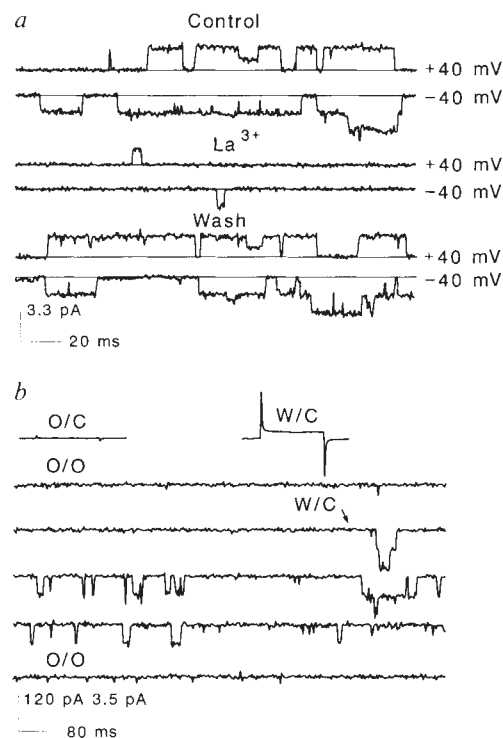


FIG. 3 *a*, Effect of La^{3+} on NMDA-gated single-channel currents. Representative traces of single-channel activity in the presence of 5 μM NMDA and 2 μM glycine (upper traces), with the addition of 5 mM La^{3+} (middle traces) and after washing out the La^{3+} (lower traces). Holding potential, -40 and $+40\text{ mV}$ in each panel, as marked. The closed state in all traces is marked by the thin line. *b*, Activity induced in an outside-out (O/O) patch by breaking from an on-cell (O/C) patch recording into a whole-cell (W/C) recording while holding the pipette potential at 0 mV . Upper left trace, response of a 10-mV voltage jump applied to the on-cell patch immediately before break-through. The resistance was about $20\text{ G}\Omega$. Upper right trace, response to a 10-mV voltage jump immediately after breaking through to the whole-cell mode. The resistance decreased to $\sim 400\text{ M}\Omega$. Lower traces, continuous recording from the hippocampal outside-out patch. Whole-cell recording from the photoreceptor commenced at a time indicated by the arrow in the second trace $\sim 50\text{ ms}$ before the first event. Bottom trace, record from the outside-out patch 820 ms after the end of the previous trace. Holding potential of the patch, -40 mV . Holding potential of the photoreceptor pipette, 0 mV . Time calibration for on-cell and whole-cell voltage-jump traces is 10 ms.

exhibited any sensitivity to applied acetylcholine at concentrations ranging from 0.1 to 10 μ M. On this basis, we conclude that all classes of turtle photoreceptors can release EAAs. These measurements do not rule out the possibility of co-release of another neurotransmitter. But, given the paucity of evidence that supports a role for GABA, acetylcholine or catecholamines,

the preponderance of results with EAA agonists and antagonists on the second-order cells^{1,2}, the demonstration of EAA-uptake mechanisms²⁰, the evidence for glutamate immunoreactivity²¹ in turtle cones, and the results of the study reported here, a strong case can be made to implicate EAAs as the photoreceptor neurotransmitter. □

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Sequence of a novel simian immunodeficiency virus from a wild-caught African mandrill

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SINCE the isolation of an HIV-2-related virus from captive macaques (SIV_{MAC})^{1,2}, the origin of human immunodeficiency viruses, a much debated subject^{3–9}, has been attributed to monkeys^{3,4}. The sequence of SIV_{AGM}, which is derived from a naturally infected African green monkey, shows equal relatedness to HIV-1 and HIV-2, suggesting that the derivation of these viruses from SIV_{AGM} is unlikely. Recent sequence analysis of SIV from a captive sooty mangabey (SIV_{SM}), however, shows its close relatedness to HIV-2 and SIV_{MAC}, indicating a possible origin of HIV-2 and SIV_{MAC} from SIV_{SM} (refs 4, 7, 9). We report here the sequence of a novel simian lentivirus, SIV_{MND}, isolated from a wild-caught mandrill in Africa¹⁰. It is distinct from the three other main groups, HIV-1, HIV-2/SIV_{MAC}/SIV_{SM} and SIV_{AGM}, and therefore represents a fourth main group of primate lentiviruses. Phylogenetic analysis indicates that these four main virus groups might have diverged from a common ancestor at about the same time, long before the spread of AIDS in humans.

We have previously isolated a simian immunodeficiency virus, SIV_{MND}, from healthy mandrills, *Papio (Mandrillus) sphinx*, from Gabon in western equatorial Africa, and shown by hybridization analysis that it is a new lentivirus differing from all known HIV and SIV group viruses. We have obtained molecular clones from extrachromosomal closed circular DNA of SIV_{MND}(GB-1), one of two SIV_{MND} isolates. A nearly full-

length clone, pSMH103, containing an insert of about 9 kb (kilobases) was used for sequencing by the Sanger method¹¹. As the pSMH103 clone lacked a small *Xba*I-*Xba*I fragment of the *pol* gene, the sequence of this fragment (nucleotides 3,536 to 3,691) was obtained from another clone of SIV_{MND}(GB-1) (pSMH21) containing a 5 kb *Sac*I-*Sac*I fragment.

The nucleotide sequence of SIV_{MND} RNA consists of 9,215 bases. In addition to the *gag*, *pol* and *env* genes, open reading frames corresponding to the *vif*, *vpr*, *tat*, *rev* and *nef* genes can be found in the SIV_{MND} genome (Fig. 1), as with the primate lentiviruses reported previously^{2,4,12–14}. This virus does not, however, contain open reading frames corresponding to *vpu*, identified in HIV-1 (ref. 13), or *vpx*, found in HIV-2 (ref. 14), SIV_{MAC}², SIV_{SM}⁴ and SIV_{AGM}¹².

The long terminal repeat (LTR) of SIV_{MND} consists of 796 base pairs (bp), and the lengths of its internal domains U3, R and U5 are estimated as 521, 175 and 100 bp, respectively. Sequences homologous to enhancers containing the κ B motif¹⁵ and Sp1 binding sites¹⁶ are found upstream of the TATA box in the U3 region. In the R region, the sequence homology of SIV_{MND} with known HIVs and SIVs is low, but the RNA transcribed between nucleotides 20 and 116 still has the potential to form the stem-loop structure of the *trans*-activation-responsive (*tar*) region¹⁷.

The *gag* open reading frame of SIV_{MND} encodes 502 amino acids and its relative molecular mass (M_r) is calculated as 56,500 (56.5K). This precursor protein may be cleaved into capsid protein, identified as p26 in SIV_{MND} virion proteins¹⁰, amino-terminal matrix protein and carboxy-terminal nucleocapsid protein. The overall sequence homologies of the *gag* precursor of SIV_{MND} to those of all the five virus isolates, BRU of HIV-1 (ref. 13), ROD of HIV-2 (ref. 14), 142 of SIV_{MAC}², F236 of SIV_{SM}⁴ and TYO-1 of SIV_{AGM}¹², are 49.2%, 48.4%, 52.0%, 50.2% and 48.2%, respectively (Table 1).

The *pol* open reading frame of SIV_{MND} with the potential to code 1,009 amino acids (115K) probably encodes the protease, reverse transcriptase and integration protein, in that order. The p66 and p55 of the reverse transcriptase and the p31 of the integration protein were identified in SIV_{MND} virion proteins¹⁰. The consensus sequence of Asp-Thr-Gly, the active site of aspartic protease, which is common to most animal retroviruses¹⁸, is also found in this virus at nucleotides 2,000–2,008. Of the open reading frames of SIV_{MND}, that of *pol* shows the highest sequence homology (56.8% to 58.2%) with those of known HIVs and SIVs (Table 1).

The *env* open reading frame of SIV_{MND} encodes a precursor that is cleaved into a surface protein (SU) and transmembrane protein (TM). The calculated molecular weights of the non-glycosylated forms of SU and TM are 59.7K and 38.6K, respec-

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TABLE 1 Amino acid homology (%) in each open reading frame

SIV _{MND} compared with:	<i>Env</i> :								
	<i>Gag</i>	<i>Pol</i>	SU*	TM†	<i>Vif</i>	<i>Vpr</i>	<i>Tat</i>	<i>Rev</i>	<i>Nef</i>
HIV-1	49.2	57.1	28.8	36.9	35.4	38.5	36.7	29.5	36.9
HIV-2	48.4	56.8	34.5	37.4	39.9	35.5	34.7	25.8	35.6
SIV _{MAC}	52.0	57.4	33.2	38.1	40.7	34.9	35.0	32.6	42.1
SIV _{SM}	50.2	57.5	33.2	40.4	39.2	38.0	36.7	34.4	40.1
SIV _{AGM}	48.2	58.2	30.0	39.5	39.4	—	42.4	31.1	39.3

Numbers indicate per cent homologies of SIV_{MND} with HIV-1(BRU)¹³, HIV-2(ROD)¹⁴, SIV_{MAC}(142)², SIV_{SM}(F236)⁴ and SIV_{AGM}(TYO-1)¹² within the aligned region using Wilbur and Lipman programs³⁴ (parameters: KTPL 2-3, DEL 3-4). * Surface, and † transmembrane proteins of *env* gene product.

tively. The TM protein is considered to be reduced in size to 29.1K by the in-frame stop codon at nucleotide 8,124, because the size of the putative TM in SIV_{MND} virion proteins is ~30K¹⁰. The in-frame stop codon in SIV_{MND} is downstream of those in HIV-2 (ref. 14), SIV_{MAC}² and SIV_{AGM}¹². There are 20 potential N-glycosylation sites on the SIV_{MND} *env* protein, 16 sites in SU and 4 in TM. Of these sites, 8 to 10 are located in the same position as those in HIV-1 (ref. 13), HIV-2 (ref. 14), SIV_{MAC}², SIV_{SM}⁴ and SIV_{AGM}¹². The homology of the SIV_{MND} *env* protein with known HIV and SIV group viruses is low; 28.8%–34.5% for SU and 36.9%–40.4% for TM (Table 1). There are 28 cysteine residues in the SIV_{MND} *env* protein, of which 18–21 are in the same position as those in HIV-1, HIV-2, SIV_{MAC}, SIV_{SM} and SIV_{AGM}. Also, 17 of them are completely conserved in all six virus groups. Based on the alignment with paramyxovirus sequences, a hydrophobic stretch at the amino terminus of TM, Phe-Leu-Gly-Phe-Leu, which could be related to the membrane fusion activity of TM, is completely conserved in all known HIV and SIV group viruses¹⁹. The corresponding sequence of SIV_{MND}, Ile-Leu-Gly-Leu-Leu, at nucleotides 7,386–7,400 is not coincident with that of other viruses, but shows a similar hydrophobic profile (data not shown). In this biologically inactive clone SMH103, the *env* and the first coding exon of *rev* are in the same phase, probably as a result of one base (C) being absent between nucleotides 5,773 and 5,776.

It is conceivable that the *tat* and *rev* proteins of SIV_{MND} are also translated for spliced subgenomic messenger RNAs, as shown in HIV-1 (refs 20, 21). In these proteins, amino-acid homology between SIV_{MND} and known HIV and SIV group viruses is low (Table 1). The seven cysteine residues encoded between nucleotides 5,579 and 5,629 in the SIV_{MND} genome, however, which are important for forming the metal-linked dimer²² of *tat* protein, are found at the same positions as those in all known HIV and SIV group viruses^{2,4,12–14}. The stretches of basic amino acid residues encoded by *tat* (corresponding to nucleotides 5,669–5,698) and *rev* (corresponding to nucleotides 8,006–8,047), which probably constitute parts of nucleic acid-binding sites²², are also found in SIV_{MND}. Also, the GTP-binding site-related sequence, demonstrated in the *nef* protein of HIV-1 (ref. 23), is also found in SIV_{MND} (nucleotides 8,515–8,601), although it shows low amino-acid homology with the sequences of other HIV and SIV group viruses.

Comparison of the genomic organization of HIV-1, HIV-2, SIV_{MAC}, SIV_{SM}, SIV_{AGM} and SIV_{MND} indicates that in addition to the *gag*, *pol* and *env* genes, *vif*, *tat*, *rev* and *nef* are common to all six virus groups and are fundamental components of primate lentiviruses. The *vpu* gene in HIV-1 (ref. 24), *vpx* in HIV-2 (ref. 25), SIV_{MAC}²⁵, SIV_{SM}⁴ and SIV_{AGM}¹², and *vpr* in HIV-1 (ref. 13), HIV-2 (ref. 14), SIV_{MAC}², SIV_{SM}⁴ and SIV_{MND},

however, are not present in all primate lentiviruses; these genes are also inessential for virus replication^{24,25}.

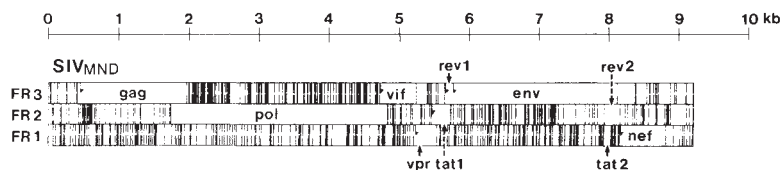
The phylogenetic tree constructed by nucleotide sequence alignment of the reverse transcriptase and integration protein in *pol* (Fig. 2) clearly indicates that the SIV_{MND} virus described here belongs to the *Lentivirinae* subfamily of retroviruses and has evolved from a common ancestral virus of primate lentiviruses just before divergence of HIV-1 from HIV-2. Although the branching order is slightly different in phylogenetic trees based on *gag* gene and other sequence comparisons (data not shown), these phylogenetic trees indicate that the four main groups of primate lentiviruses, SIV_{MND}, SIV_{AGM}, HIV-2/SIV_{MAC}/SIV_{SM} and HIV-1, diverged from a common ancestral virus at about the same time.

SIV_{MAC} and SIV_{SM} are genetically very close to HIV-2 (refs 2, 4), and recent reports^{26,27} indicate considerable genetic variability in HIV-2 isolates. Therefore, SIV_{MAC}, SIV_{SM} and HIV-2 can be considered as one virus group, as shown in Fig. 2. Large scale serological surveys of non-human primates^{28,29} has shown that many macaques in natural conditions are seronegative, whereas the observed sooty mangabeys (*Cercocebus atys*), indigenous to West Africa, were seropositive for SIV_{MAC}^{3,29}. From this observation, Hirsch *et al.*⁴ suggested that SIV_{SM}-infected macaques in captivity, and humans in West Africa, evolving as SIV_{MAC} and HIV-2, respectively. As the SIV_{SM} isolate sequenced originated from a captive animal in the Delta Primate Center in the United States, however, it is necessary to determine the sequence of SIV obtained from a sooty mangabey in the wild.

Several recent studies on molecular evolution have suggested that the *gag* and *pol* genes of HIV-1 have evolved at approximate rates of 10⁻³ nucleotide substitutions per site per year^{30–32}. By extrapolation of these substitution rates to the time of evolution of all primate lentiviruses, we estimate that the four main lentivirus groups diverged several hundred years ago. These substitution rates, however, are based on a comparison of multiple isolates within only the HIV-1 group^{30–32}, and so are not directly applicable to all primate lentiviruses, which are highly divergent in species specificity and pathology.

The SIV_{MND}(GB-1) isolate sequenced in this study originated from a wild mandrill, because the animal was already seropositive when it was brought to Franceville Primate Center in Gabon immediately after its capture in the wild¹⁰. The possibility that this mandrill was infected by other monkey species in the wild is unlikely; other infected mandrills, although in a zoo²⁹, have been reported that were probably obtained from other places in Africa. Thus mandrills are almost certainly infected with SIV_{MND} in the wild. This virus apparently does not cause disease in mandrills, as in the case of SIV_{AGM}, although only a small number have been examined so far^{28,29}. The SIV_{AGM}(TYO-1)

FIG. 1 Organization of the SIV_{MND} genome as determined from the sequence. Vertical bars represent stop codons in the three reading frames (FR1–FR3). Arrows indicate the potential AUG initiator codon of each open reading frame. *Tat* 1 and 2, and *rev* 1 and 2 are open reading frames containing possible coding exons of the *tat* and *rev* genes.



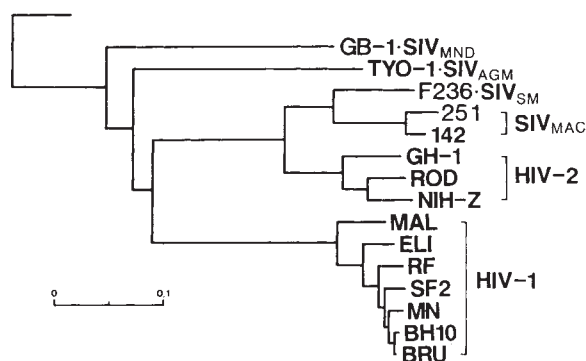


FIG. 2 Phylogenetic tree showing the evolutionary relationship of SIV_{MND} and known primate lentiviruses. The tree was constructed by the neighbour-joining method³⁵ after alignment of the nucleotide sequences of the reverse transcriptase and the integration protein in the *pol* gene. The viral sequences used in this study were as follows: GB-1 isolate of SIV_{MND} in this study; TYO-1 isolate of SIV_{AGM}¹²; Delta/F236 isolate of SIV_{SM}⁴; 251³⁶ and 142² isolates of SIV_{MAC}; GH-1²⁷, ROD¹⁴ and NIH-Z²⁶ isolates of HIV-2; MAL³⁷, ELI³⁷, RF³⁸, SF2³⁹, MN⁴⁰, BH10⁴¹ and BRU¹³ isolates of HIV-1. The sequences of ovine visna lentivirus (VISNA)⁴² and equine infectious anaemia virus (EIAV)⁴³ were used as reference species of lentiviruses in the calculation to make the position of the root in the tree clear. Horizontal distances from each branch are proportional to the number of amino-acid substitutions. The scale bar indicates 0.1 substitutions per site.

isolate can be considered as a representative strain of SIV_{AGM}, which is highly endemic in African green monkeys in the wild^{12,28,33}. Our sequence analysis of SIV_{MND} and SIV_{AGM} suggests that several species of African non-human primates have harboured SIV for a long time and that these SIVs are specific to their natural hosts. □

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Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis

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T CELLS expressing the $\alpha\beta$ T-cell receptor (TCR) for antigen can elicit anti-idiotypic antibodies specific for the TCR that regulate T-cell function^{1–4}. Defined sequences of the TCR, however, have not been used to elicit specific antibodies and the role of cellular immunity directed against TCR determinants has not been studied. We immunized Lewis rats with a synthetic peptide representing a hypervariable region of the TCR V β 8 molecule. Subsequent induction of experimental autoimmune encephalomyelitis, a paralytic disease of the central nervous system mediated primarily by V β 8⁺ T cells specific for myelin basic protein^{5,6} was prevented. T cells specific for the TCR V β 8 peptide conferred passive protection against the disease to naive rats, apparently by shifting the predominant T-cell response away from the major encephalitogenic epitope of basic protein. This is the first report demonstrating the use of a synthetic TCR V-region peptide to induce specific regulatory immunity and has important implications for the regulation of human disease characterized by common TCR V-gene usage.

In a previous study⁶, we reported the complete nucleotide and deduced amino-acid sequence for the rearranged rat TCR α - and β -chain genes (with sequence homology to the mouse V α 2 and V β 8 families respectively) used in response to the major encephalitogenic epitope of basic protein, namely residues 72–89. Within the TCR V β 8 region, we identified and synthesized a 21-amino acid sequence that included the second complementarity determining region (CDR2) and was predicted to be immunogenic for T cells^{7,8}. A control peptide was synthesized from the corresponding region of a different TCR V β sequence that was homologous to the mouse V β 14 family⁹.

Immunization of Lewis rats with the TCR V β 8 peptide but not with the TCR V β 14 peptide or saline prevented completely the induction of experimental autoimmune encephalomyelitis (EAE) (Table 1). The V β 8-peptide-immunized rats developed both specific antibodies [0.63 optical density (OD) units to the V β 8 peptide compared with 0.02 OD units to the control peptide at a 1:200 dilution of serum analysed by enzyme-linked immunosorbent assay (ELISA)] and delayed type hypersensitivity (DTH) responses 17/100 mm = 0.17 mm ear swelling to 50 μ g TCR V β 8 peptide compared with 0/100 mm = 0.00 mm swelling to the control peptide. The V β 14 peptide also induced specific immunity in rats injected with the V β 14 peptide even though it did not confer protection against EAE. Lymph node cells isolated from the protected rats responded to the TCR V β 8 peptide as well as to guinea-pig basic protein (GPBP) and PPD (purified protein derivative of *Mycobacterium tuberculosis*) (Table 2).

T-cell lines were selected from the lymph nodes of the protected rats that responded specifically to the V β 8 but not to the V β 14 peptide (Table 2). The TCR V β 8 peptide-specific T cells

TABLE 1 Immunization with TCR V β 8-peptide prevents induction of EAE

Immunization protocol	Incidence	Day of onset	EAE Induction*	Duration	Severity†
TCR V β 8 peptide + CFA‡	0/10	—	—	—	—
GPBP + CFA after 30 days					
TCR V β 14 peptide + CFA§	4/4	14 $\pm$ 2		6 $\pm$ 2	3.1 $\pm$ 0.3
GPBP + CFA after 30 days					
Saline + CFA	14/14	14 $\pm$ 2		6 $\pm$ 1	3.0 $\pm$ 0.5
GPBP + CFA after 30 days					

* EAE was induced by subcutaneous injection of 50 μ g GPBP + 400 μ g *Mycobacteria* in complete Freund's adjuvant (CFA).

† Value represents the mean of the maximum severity of EAE. 0, no signs; 0.5, lethargy, weight loss; 1, limp tail; 2, hind-leg weakness; 3, hind-quarter paralysis, incontinence; 4, moribund.

‡ 100 μ g of the TCR peptide [DMGHGLRLIHYSDVNSTK (single-letter code)] representing residues 39–59 of the rat cDNA clone V β 510 (ref. 6) which has homology with the mouse V β 8 family was mixed with CFA containing 100 μ g *Mycobacteria* and injected subcutaneously.

§ 100 μ g of TCR peptide [APGGTLQQLFYFNVGQSELV] representing residues 39–59 of the rat cDNA clone CRTB188 (ref. 9) which has homology with the mouse V β 14 family was mixed with CFA containing 100 μ g *Mycobacteria* and injected subcutaneously.

TABLE 2 Specificity of T-cell lines from rats protected from EAE by V β 8 peptide

Stimulant	LN	Proliferation (c.p.m. $\times 10^{-3}$)		TCR V β 8 line + GPBP line
		TCR V β 8 peptide selected line	GPBP selected line	
Medium	11 $\pm$ 1	2 $\pm$ 1	1 $\pm$ 0	6 $\pm$ 1
Con A	99 $\pm$ 6	96 $\pm$ 4	80 $\pm$ 4	123 $\pm$ 5
TCR V β 8 peptide	27 $\pm$ 3	100 $\pm$ 6	1 $\pm$ 0	140 $\pm$ 4
+ OX-6 (anti I-A)	ND	99 $\pm$ 8	ND	ND
+ OX-17 (anti I-E)	ND	100 $\pm$ 4	ND	ND
+ OX-18 (anti class I)	ND	31 $\pm$ 5	ND	ND
+ W3/25 (anti-CD4)	ND	105 $\pm$ 7	ND	ND
+ OX-8 (anti-CD8)	ND	22 $\pm$ 3	ND	ND
TCR V β 14 peptide	10 $\pm$ 1	2 $\pm$ 0	ND	ND
GPBP	16 $\pm$ 1	3 $\pm$ 1	93 $\pm$ 6	102 $\pm$ 5
1–37	13 $\pm$ 1	ND	1 $\pm$ 1	13 $\pm$ 2
44–89	20 $\pm$ 1	ND	46 $\pm$ 12	56 $\pm$ 1
44–68	15 $\pm$ 1	1 $\pm$ 0	2 $\pm$ 0	22 $\pm$ 5
72–89	16 $\pm$ 1	1 $\pm$ 0	27 $\pm$ 3	20 $\pm$ 2
+ OX-6	ND	ND	1 $\pm$ 0	ND
+ OX-17	ND	ND	23 $\pm$ 2	ND
+ OX-18	ND	ND	27 $\pm$ 1	ND
+ W3/25	ND	ND	1 $\pm$ 0	ND
+ OX-8	ND	ND	26 $\pm$ 2	ND
87–99	12 $\pm$ 1	ND	1 $\pm$ 0	ND
90–170	13 $\pm$ 3	ND	1 $\pm$ 0	14 $\pm$ 3
GPBP + V β 8 peptide	30 $\pm$ 2	ND	55 $\pm$ 9	149 $\pm$ 9
PPD	48 $\pm$ 3	2 $\pm$ 0	1 $\pm$ 1	ND
Rt-BP	11 $\pm$ 2	1 $\pm$ 0	9 $\pm$ 2	9 $\pm$ 1

Rats were immunized subcutaneously (s.c.) with 400 μ g TCR V β 8 peptide in CFA containing 1 mg *M. tuberculosis*, and were challenged s.c. with 50 μ g GPBP in CFA at the same time or with 100 μ g encephalitogenic peptide after 30 days. Twenty days after simultaneous challenge, draining lymph nodes were removed and a single-cell suspension was tested in microtitre wells (5×10^5 cells per well) for response to antigens and mitogens. The remainder of the cells were stimulated in 6-cm diameter Petri dishes with either TCR peptide or GPBP (50 μ g ml $^{-1}$) for 3 days and transferred into interleukin-2 (IL-2) rich medium for an additional 4 days. These cells (2×10^4 per well) were restimulated with antigens and mitogens in the presence of irradiated thymic accessory cells. Stimulation in the presence of TCR peptide or GPBP was carried out in the presence of 2 μ g per well monoclonal antibodies. Underlined values indicate significant stimulation. ND, not done.

TABLE 3 Response of TCR V β 8 peptide-specific T-cell line to attenuated V β 8 (+) or (–) T cells

Stimulator line (specificity)	V β 8 expression	Proliferation (c.p.m. $\times 10^{-3}$)
None		7 $\pm$ 2
GPBP (S72–89)*	+	31 $\pm$ 3
GPBP (S5–74)*	–	8 $\pm$ 1

T-cell-line cells were irradiated (2,500 R) and 2×10^4 cells were mixed in the absence of additional accessory cells with 2×10^5 TCR specific T cells for 3 days, the last 18 h with [3 H]Thy. The cells were harvested and [3 H]Thy uptake assessed by liquid scintillation. Background proliferation of irradiated T cells specific for GPBP (S72–89) and GPBP (S5–74) was 130 and 220 c.p.m. respectively.

* TCR V gene use for these T-cell lines was described previously¹¹.

were strongly positive by immunofluorescence for the CD4 marker and weakly positive for the CD8 marker, but the response to the V β 8 peptide was restricted only by MHC class I molecules.

A GPBP-specific T-cell line was also selected from protected rats immunized with both the TCR V β 8 peptide and GPBP. This line had an uncharacteristically low response to the encephalitogenic 72–89 peptide relative to GPBP¹⁰. Once selected and activated, GPBP-specific T-cell-line cells from TCR-peptide-protected rats were encephalitogenic (hind-leg paralysis with 10 million cells in all 3 rats), indicating that V β 8 peptide immunization did not result in the deletion of encephalitogenic T-cell precursors.

Mixing of the TCR V β 8-specific and BP-specific T cells did not impair the response to GPBP, even in the presence of the TCR peptide (Table 2). The presence of TCR V β 8-specific T cells, however, caused, an increased response to all of the

TABLE 4 TCR V β 8 peptide-specific T-cells protect against EAE

Transfer dose*	Incidence	Induction of EAE (GPBP/CFA)		Severity†	DTH (mm $\times 10^{-2}$)	
		Day of onset	Duration		GPBP	PPD
None	5/5	12	6.5	3.1	32	21
3×10^7	0/5	—	—	0	24	21
1×10^7	0/3	—	—	0	ND	ND

* T-cell line cells were stimulated with TCR V β 8 peptide plus thymic accessory cells for 3 days prior to intraperitoneal transfer into naive recipient rats. The rats were challenged on the same day with GPBP/CFA.

† Value represents the mean of the maximum severity of EAE. See Table 1 legend.

peptides of GPBP except the encephalitogenic 72–89 sequence. The TCR peptide-specific T cells therefore seemed to alter the peptide recognition pattern of GPBP reactive T cells and indicated the possibility of cell–cell interactions.

Direct interactions could also be demonstrated. The addition of irradiated V β 8⁺ target cells induced significant proliferation by the TCR-specific T-cell line in the absence of additional accessory cells, whereas V β 8[−] T cells¹¹ did not (Table 3), indicating the direct recognition of the TCR sequence on the target T-cell surface. The TCR peptide-specific T cells, however, were not cytotoxic for the BP-reactive target cells (data not shown).

The protective ability of V β 8 peptide-specific T cells was established by adoptive transfer. Rats injected with as few as 10 million V β 8 peptide-specific T cells did not develop EAE (Table 4). The transferred protection appeared to be T-cell mediated, as we were unable to detect V β 8 peptide-specific antibodies in the serum of protected rats. DTH results (Table 4) indicated that the adoptively transferred T cells could prevent the induction of EAE without compromising T-cell recognition of other antigens.

The ability of V β 8-peptide-specific T cells to alter the response patterns of GPBP specific T-cell lines *in vitro* and to protect naive rats from EAE and reduce DTH reactions *in vivo* indicated that the pattern of response to BP epitopes might be altered in rats protected by TCR-peptide-specific T cells. As shown in

Table 5, lymph-node cells (LNC) from the EAE-protected animals responded well to the TCR peptide, but poorly to GPBP and the encephalitogenic 72–89 peptide of GPBP compared to LNC from the control group. By contrast, LNC from the protected group showed a significant response to the 87–99 peptide of BP, whereas LNC from the control group had no response to this peptide (Table 5). The selection of a TCR-V β 8-peptide-specific T-cell line from the lymph nodes of adoptively protected rats (Table 5) indicated that TCR-peptide-specific T cells had migrated to and persisted in the lymph nodes that drained the site of GPBP injection.

These results demonstrate for the first time the use of a synthetic peptide from the CDR2 region of the TCR to induce V β 8-specific regulatory T cells that prevent the induction of EAE. Computer modelling of ternary interactions among TCR chains, antigenic peptides, and MHC restriction molecules is consistent with CDR involvement in peptide/MHC binding when the TCR is folded in an energetically favourable conformation^{12,13}. The indisputable regulatory effects of T cells specific for CDR2 support the notion that this region has biological importance.

It seems unlikely that the responder T cell is interacting directly with the functional TCR V β 8 molecule on the target T-cell surface. Indeed, it is conceivable that endogenous TCR peptide could be 'processed' and expressed preferentially on the T-cell surface in association with class I molecules¹⁴. If a natural form of the TCR peptide is associated with the MHC on the T-cell surface, the interacting TCR-specific T cell could interfere with normal activation by BP.

Vaccination with attenuated T cells has provided indirect evidence that protection against autoimmunity may include T-cell recognition of TCR^{15–20}. These data indicated that protective immunity was induced against target structures shared by different T-cell clones specific for the same disease-inducing epitope, but did not implicate the TCR directly. The immunogenicity and immunoregulatory activity demonstrated here of a defined region of the TCR V β 8 chain expressed by encephalitogenic T cells is an important step forward in understanding anti-idiotypic regulation, and provides a clear explanation for the protective effects of the vaccination approach.

The induction of anti-idiotypic antibodies directed against TCR determinants expressed on the surface of intact T cells has played a crucial role in the identification and function of the TCR itself^{1–4}. Two of the TCR V β 8 specific monoclonal antibodies, F23.1 and KJ16, can inhibit EAE in the mouse, which also uses the TCR V β 8 gene in response to BP^{21,22}. Our approach using a synthetic peptide to induce TCR-peptide-specific antibodies may be of value in producing a variety of highly specific antibodies for assessing sequences important in TCR function. The potential regulatory properties of antibodies raised to the V β 8 peptide will be addressed in a separate study.

TCR peptide vaccination may have application in human autoimmune or malignant conditions that are characterized by common TCR V-gene usage. This approach would seem to have clear advantages over other approaches such as tolerance induced with potentially pathogenic target autoantigens, or whole-cell vaccination which is 'individual' specific and complicated by the presence of extraneous cell-surface antigens. □

TABLE 5 Specificity of T-cell lines from rats protected from EAE by V β 8 peptide-specific T cells.

Protocol	Stimulant	Proliferation (c.p.m. $\times 10^{-3}$)		
		LN	TCR peptide selected line	GPBP selected line
3×10^7 V β 8 peptide-specific T cells	Medium	7 $\pm$ 1	2 $\pm$ 1	5 $\pm$ 1
	Con A	<u>125 $\pm$ 14</u>	<u>46 $\pm$ 14</u>	<u>39 $\pm$ 1</u>
	V β 8 peptide	<u>90 $\pm$ 6</u>	<u>52 $\pm$ 3</u>	6 $\pm$ 1
	GPBP	8 $\pm$ 0	0 $\pm$ 0	<u>16 $\pm$ 4</u>
	1–37	7 $\pm$ 2	ND	6 $\pm$ 1
	44–89	<u>12 $\pm$ 2</u>	ND	<u>17 $\pm$ 2</u>
	44–68	<u>9 $\pm$ 1</u>	4 $\pm$ 3	<u>9 $\pm$ 1</u>
	72–89	<u>12 $\pm$ 3</u>	3 $\pm$ 1	<u>11 $\pm$ 1</u>
	87–99	<u>41 $\pm$ 7</u>	4 $\pm$ 2	<u>9 $\pm$ 1</u>
	90–170	<u>12 $\pm$ 2</u>	ND	<u>8 $\pm$ 1</u>
Saline, GPBP + CFA	Medium	12 $\pm$ 1	Not selected	6 $\pm$ 1
	Con A	<u>38 $\pm$ 4</u>		<u>88 $\pm$ 10</u>
	V β 8 peptide	8 $\pm$ 1		6 $\pm$ 2
	GPBP	<u>32 $\pm$ 5</u>		<u>65 $\pm$ 2</u>
	1–37	<u>12 $\pm$ 0</u>		6 $\pm$ 1
	44–89	<u>34 $\pm$ 6</u>		<u>91 $\pm$ 5</u>
	44–68	<u>19 $\pm$ 3</u>		<u>24 $\pm$ 3</u>
	72–89	<u>29 $\pm$ 2</u>		<u>64 $\pm$ 5</u>
	87–99	<u>11 $\pm$ 1</u>		6 $\pm$ 0
	90–170	<u>11 $\pm$ 2</u>		7 $\pm$ 1

Lymph-node cells were collected and tested 20 days after simultaneous injection of TCR V β 8 peptide-specific T line cells (or saline) and GPBP/CFA. T cell lines were selected from the LNC as described in Table 2. Underlined values indicate significant stimulation.

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Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*

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IN antibodies, a heavy and a light chain variable domain, VH and VL, respectively, pack together and the hypervariable loops on each domain contribute to binding antigen^{1–4}. We find, however, that isolated VH domains with good antigen-binding affinities can also be prepared. Using the polymerase chain reaction⁵, diverse libraries of VH genes were cloned from the spleen genomic DNA of mice immunized with either lysozyme or keyhole-limpet haemocyanin. From these libraries, VH domains were expressed and secreted from *Escherichia coli*. Binding activities were detected against both antigens, and two VH domains were characterized with affinities for lysozyme in the 20 nM range. Isolated variable domains may offer an alternative to monoclonal antibodies and serve as the key to building high-affinity human antibodies. We suggest the name 'single domain antibodies (dAbs)' for these antigen binding demands.

We have analysed the interactions with antigen of individual domains of the anti-lysozyme antibody, D1.3 (ref. 1). The VH domain was expressed in *E. coli* and secreted into the periplasm^{6,7}, alone or in association with the Vκ domain (fig. 1). Analysis of culture medium, by passage through a lysozyme-Sepharose affinity column⁸, followed by SDS-PAGE⁹ revealed that both the isolated VH domain, or the associated Fv fragment, could bind to lysozyme, and could be purified to homogeneity in a single step, with yields of ~200 µg l⁻¹ and 10 mg l⁻¹, respectively. The VH domain appears to be monomeric by FPLC (Pharmacia, Superose 12 column). The N-terminal sequences of both domains were checked by gas-phase protein sequencing^{10,11}.

As shown in Table 1, the affinity of Fv fragment for lysozyme and the stoichiometry of binding of the VH domain to lysozyme were determined by titration using fluorescence quench techniques. The affinity of VH domain for lysozyme was determined

TABLE 1 Affinities of Fv fragment and VH domains for hen egg lysozyme

	Stoichiometry	Affinity (nM)	k_{on} (M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	k_{off}/k_{on} (nM)
Fv-D1.3	ND	3	1.9×10^6	ND	ND
VH-D1.3	1.2	ND	3.8×10^6	0.075	19
VH3	ND	ND	2.9×10^6	0.036	12
VH8	ND	ND	3.3×10^6	0.088	27

Cultures of 500 ml were grown and induced (see Fig. 1 methods), and the supernatant passed through a 0.45 µm filter (Nalgene), then through a 5 ml lysozyme-Sepharose affinity column. After washing with phosphate buffered saline (PBS), the Fv fragment or VH domains were eluted with 50 mM diethylamine, and analysed for purity by SDS-PAGE⁹. The proteins were titrated with lysozyme at 25 °C using fluorescence quench (Perkin Elmer LS 5B Luminescence Spectrometer)²⁷ to determine the number of active binding sites, to measure the affinity of the Fv fragment and the stoichiometry of binding of the VH domain (mole lysozyme per mole domain). The concentration of the VH domain of the D1.3 antibody was determined by hydrolysis followed by quantitative amino-acid analysis. The kinetics of lysozyme binding were determined by stopped-flow (Hi Tech Stop Flow SHU) at 20 °C under pseudo-first order conditions with binding sites in five to ten fold excess over lysozyme²⁸. For the kinetics, the concentration of binding sites, not protein, was measured ND, not determined. k_{on} is the second order rate constant for association, and k_{off} is the first order rate constant for dissociation.

from the kinetics of binding. The affinity of the Fv fragment (3 nM) is similar to the parent antibody (2 nM). The VH domain binds lysozyme tightly in an equimolar complex with an affinity for lysozyme (19 nM) which is only 10-fold weaker. Separated heavy and light chains have previously been identified with antigen¹² or hapten binding activities¹³ although the affinities were poor, with no evidence for binding by single chains^{13,14} rather than dimers¹⁵.

In the D1.3 antibody, lysozyme interacts extensively with both domains, and forms three hydrogen bonds to the Vκ domain, and nine hydrogen bonds to the VH domain. Binding of lysozyme buries ~300 Å² of Vκ domain away from solvent, and 400 Å² of the VH domain¹. Our results show, however, that the Vκ domain makes only a small net contribution to the energetics of binding. This is surprising as the removal of a single hydrogen bond¹⁶ or a single van der Waals contact¹⁷ can lead to tenfold loss in affinity. The VH domain presumably binds to lysozyme in a similar way to the antibody and this is consistent with inhibition of binding of the Fv fragment by the VH domain (data not shown, but see Fig. 1 legend). It is possible that the whole surface of interaction might reorientate slightly, perhaps by rocking on side chains, to create a new set of contacts¹⁸.

The result prompted us to obtain VH domains with antigen-binding activities from antibody-producing cells. Previously we have demonstrated the cloning of immunoglobulin variable regions from hybridoma mRNA for expression of chimaeric antibodies, using the polymerase chain reaction (PCR)^{5,19}. We now used PCR to amplify the rearranged VH genes from the spleen DNA of a mouse immunized with lysozyme (Fig. 2). The amplified DNA was cloned into the vector M13VHPCR1 (ref. 19) for sequencing. The complete sequences of 48 VH gene clones were determined (data not shown). All but two of the mouse VH gene families²⁰ were represented, with frequencies of: VA (1), IIIC (1), IIIB (8), IIIA (3), IIB (17), IIA (2), IB (12) and IA (4). In 30 clones the D segments could be assigned to families SP2 (14), FL16 (11) and Q52 (5), and in 38 clones the JH minigenes to families JH1 (3), JH2 (7), JH3 (14) and JH4 (14). The different sequences of CDR3 marked each of the 48 clones as unique. Nine pseudogenes and 16 unproductive rearrangements were identified; of the clones sequenced, 27 have open reading frames. Clearly we can generate a diverse repertoire of VH genes using PCR, but cannot rule out a systematic bias due to our choice of primers and hybridization conditions. VH gene libraries have also been generated using PCR

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FIG. 1 Vectors for expression of VH and V κ domains. Scheme showing inserts in expression vectors. a, pSW1-VHD1.3; b, pSW1-VHD1.3-VKD1.3; c, pSW1-VHD1.3-TAG1; d, pSW1-VHD1.3-VKD1.3-TAG1; e, pSW1-VHPOLY; f, pSW1-VHPOLY-TAG1; and g, nucleotide sequence of pSW1-VHPOLY-TAG1 insert. The amino acid sequence of the pelB leader and TAG1 are shown in italics.

METHODS. The vectors were assembled from pUC19 (ref. 29), synthetic oligonucleotides encoding the pelB signal sequence³⁰ peptide tag²⁴, restriction site polylinker, and (as appropriate) cloned cDNA of the VH and V κ domains of the D1.3 antibody (M. E. Verhoeven, C. Berek and G. W., unpublished data). Recombinant plasmids were transformed into *E. coli* BMH71-18 (ref. 31), colonies selected on TYE plates³² with 100 μ g ml⁻¹ ampicillin (AMP) and 1% glucose (GLU), and toothpicked into 200 μ l 2 $\times$ TY medium³², AMP, GLU in wells of ELISA plates. Colonies were grown at 37 °C for 16–24 h. Cells were pelleted, washed twice in 50 mM NaCl (200 μ l) and resuspended in 200 μ l 2 $\times$ TY medium, AMP and 1 mM isopropylthiogalactoside (to induce expression) and grown for a further 16–24 h. The cells were cooled, pelleted and supernatants screened for secretion of VH domains (by western blotting) or antigen binding activity (by direct ELISA). Western blot methods were essentially as in ref. 22: supernatant (10 μ l) from the cultures was subjected to SDS-PAGE⁹ and proteins then transferred electrophoretically to nitrocellulose. The VH domains were detected by means of the peptide tag with 9E10 antibody²⁴. However, the tag can be lost by proteolytic cleavage in culture, especially after prolonged growth of the bacteria. Bound antibody was detected using horseradish peroxidase conjugated rabbit anti-mouse antibody at a dilution of 1:1000. 4-chloro-1-naphthol (Sigma) was used as the peroxidase substrate. For direct ELISA, wells of Falcon ELISA plates were coated with antigen in phosphate buffered saline (PBS) overnight (3 mg ml⁻¹ lysozyme, or 50 μ g ml⁻¹ KLH), then blocked with 2% skimmed milk powder in PBS for 2 h at 37 °C. Bacterial supernatant was added and incubated at 37 °C for 2 h. D1.3-VH domains were detected with rabbit polyclonal antiserum raised against the D1.3 Fv fragment, using peroxidase conjugated goat anti-rabbit immunoglobulin. Tagged VH domains were detected as described in western blotting except with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) as the peroxidase substrate. Three washes of 0.05% Tween 20 in PBS, were followed by three washes of PBS between each step (only PBS washes before addition of blocker or bacterial supernatants). Competition ELISA was as above except that the binding of the Fv fragment tagged on the V κ domain was competed against by untagged VH domain.

from mRNA of human peripheral blood lymphocytes (J. Marks, D.G. & G.W., unpublished data) and from mRNA of mouse spleen²¹.

Amplified DNA was then cloned for expression into a vector which incorporates a C-terminal peptide tag to facilitate detection of expressed VH domains (Fig. 1f). Bacterial supernatants were analysed by SDS-PAGE followed by western blotting²², and bands of the expected size ($M_r \approx 14,000$) were detected for 14 of the 17 clones by probing with antibody directed against the tag^{23,24}. To screen for lysozyme binding activities, about two thousand colonies were toothpicked in groups of five into wells of enzyme-linked immunosorbent assay (ELISA) plates, and the supernatants tested for binding to lysozyme-coated plates. Twenty-one supernatants were shown to have lysozyme-binding activity, and some of the corresponding individual clones were prepared.

Two of the clones (VH3 and VH8) with lysozyme-binding activities were sequenced (Fig. 3). They belonged to the same VH-gene (Kabat IIB) families and D-segment families (FL16) but had different J segments (JH2 and JH4). There were only six amino-acid differences between the (unrearranged) VH genes, but the sequences of CDR3 were completely different. The VH domains were purified and affinities for lysozyme determined (Table 1). The affinities, in the 20 nM range, are similar to those of the VH domain of the D1.3 antibody. To check the specificity of binding, the three VH domains were also screened for binding to four other purified proteins (bovine serum albumin, insulin, keyhole-limpet haemocyanin (KLH) and cytochrome c), and to foetal calf serum, milk powder and plastic of microtitre plates. No binding was detected. (However, other cross-reactive VH domains have been found.) The VH-D1.3, VH3 and VH8 domains appear to bind to the same region of lysozyme, as they inhibit the binding of the D1.3 Fv fragment.

To test whether VH domains with other binding activities

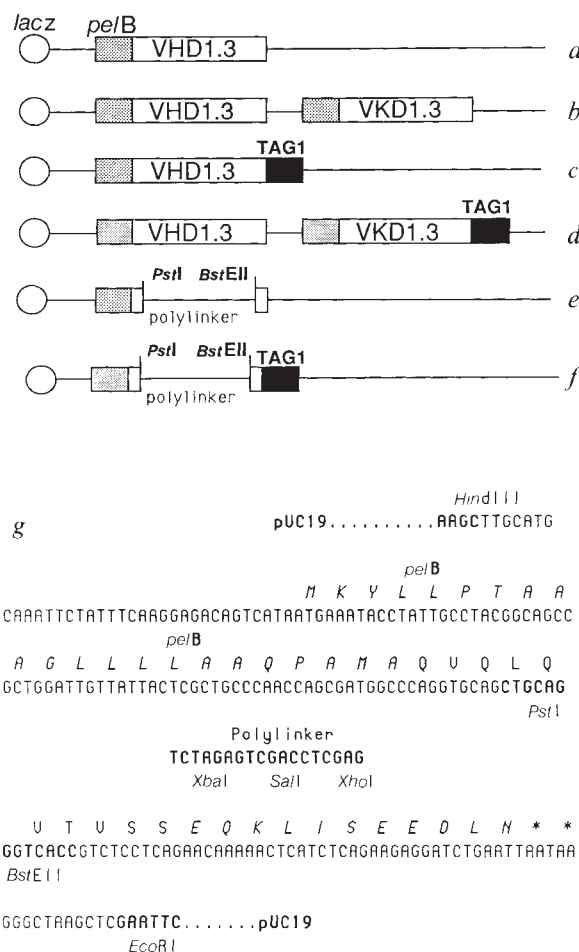


FIG. 2 PCR amplification of VH genes from mouse genomic DNA. Agarose gel electrophoresis of amplified mouse VH DNA (left-hand lane) with markers OX174 HaeIII fragments (right-hand lane). **METHODS.** Balb/c mice were hyperimmunized with hen egg-white lysozyme (100 μ g antigen, intraperitoneally on day 1 in complete Freund's adjuvant and on day 14 in incomplete Freund's adjuvant; followed by 50 μ g antigen intravenously on day 35; mice were killed on day 39) or similarly with KLH. DNA was prepared from the spleen and the rearranged mouse VH genes were amplified. Conditions were chosen to minimize annealing between the 3' ends of the two primers. The sample (50–100 μ l) included 50–200 ng DNA, VH1FOR-2 (5' TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3') and VH1BACK primers¹⁹ (25 pm of each) 250 μ M of each dNTP, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 100 μ g ml⁻¹ gelatine. The sample was overlaid with paraffin oil, heated to 95 °C for 2 min, 65 °C for 2 min, and then to 72 °C; Taq polymerase (2 units, Cetus) was added after the sample had reached the elongation temperature and the reaction continued for 2 min at 72 °C. The sample was subjected to a further 29 rounds of temperature cycling using the Techne PHC-1 programmable heating block. The amplified DNA was digested with PstI and BstEII and fractionated on an agarose gel. A band of about 350 base pairs was extracted and cloned.

could be made, and whether immunization was necessary, a new VH-gene library was prepared from a mouse immunized with KLH. Culture supernatants from the two libraries were tested for binding to lysozyme or to KLH. The first library (immunization with lysozyme) had yielded 21 supernatants with lysozyme- and two with KLH-binding activities, whereas the second library (immunization with KLH, screening ~2,000

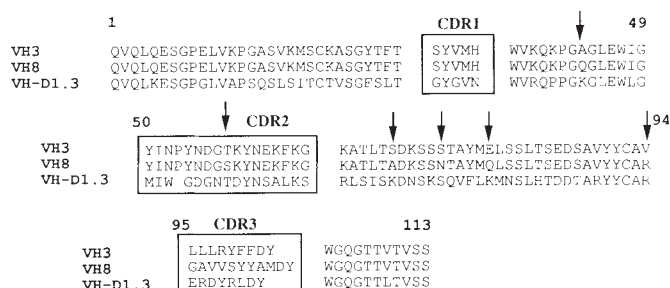


FIG. 3 Sequences of VH domains with lysozyme-binding activities. The sequences of the VH domains are aligned with that of the D1.3 antibody.

colonies) yielded two supernatants with lysozyme- and 14 with KLH-binding activities. We conclude that VH domains can be derived, preferably after immunization, with binding activities to lysozyme and KLH and presumably other antigens. VH domains lack the cavity which can be formed with the V_K partner however, and this might bias the binding activities against hapten binding^{2,15}. The affinity of the VH domains (20 nM or $5 \times 10^7 \text{ M}^{-1}$) for lysozyme lies within the range expected for the affinities of monoclonal antibodies for protein antigens, and can be improved by site-directed mutagenesis (unpublished data).

VH domains with binding activities can be generated in a matter of days without recourse to tissue culture, and may also have other advantages over monoclonal antibodies. For example, the smaller molecule should penetrate tissues more readily, could permit the blocking of 'canyon' sites on viruses^{25,26} and allow epitope mapping at higher resolution. However, VH domains are relatively 'sticky', presumably due to the exposed hydrophobic surface normally capped by the V_K or V_L domains. It should be possible to design VH domains with improved properties. We also envisage that VH domains with binding activities could serve as the building blocks for making Fv fragments or complete antibodies. For example, such VH domains could be co-expressed with a repertoire of V_K domains, derived by PCR amplification of V_K genes¹⁹ and screened for association of the domains and antigen binding. This approach could prove valuable for building human antibodies of therapeutic value, and even for catalytic antibodies; for example, making Fv fragments in which the VH domain binds substrate, and side chains or prosthetic groups in the V_K partner stabilize the transition state or attack the substrate³³. □

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Protease nexin-II, a potent anti-chymotrypsin, shows identity to amyloid β -protein precursor

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PROTEASE nexin-II (PN-II) is a protease inhibitor that forms SDS-resistant inhibitory complexes with the epidermal growth factor (EGF)-binding protein, the γ -subunit of nerve growth factor, and trypsin¹⁻³. The properties of PN-II indicate that it has a role in the regulation of certain proteases in the extracellular environment. Here we describe more of the amino-acid sequence of PN-II and its identity to the deduced sequence of the amyloid β -protein precursor (APP)^{4,5}. Amyloid β -protein is present in neuritic plaques and cerebrovascular deposits in individuals with Alzheimer's disease and Down's syndrome⁶⁻⁹. A monoclonal antibody against PN-II (designated mAbP2-1) recognized PN-II in immunoblots of serum-free culture medium from human glioblastoma cells and neuroblastoma cells, as well as in homogenates of normal and Alzheimer's disease brains. In addition, mAbP2-1 stained neuritic plaques in Alzheimer's disease brain. PN-II was a potent inhibitor of chymotrypsin with an inhibition constant K_i of $6 \times 10^{-10} \text{ M}$. Together, these data demonstrate that PN-II and APP are probably the same protein. The regulation of extracellular proteolysis by PN-II and the deposition of at least parts of the molecule in senile plaques is consistent with previous reports that implicate altered proteolysis in the pathogenesis of Alzheimer's disease^{4,5,10-12}.

Protease nexins are protein protease inhibitors that are synthesized and secreted by various cultured extravascular cells. They form SDS-resistant complexes with certain serine proteases; the complexes then bind back to the cells and are rapidly internalized and degraded^{1-3,13,14}. Protease nexin-I is a specific thrombin inhibitor when bound to the cell surface¹⁵. It stimulates neurite outgrowth in cultured neuroblastoma cells^{16,17} and primary chick sympathetic neurons¹⁸ and is reduced by about six-fold in Alzheimer's disease brain¹⁹. PN-II is unusually stable and forms complexes with target proteases even after incubation of the protein with SDS or at a pH of 1.5 (ref. 3).

We analysed the N-terminal amino-acid sequence of two peptides obtained from digestion of PN-II. Sequencing of a CNBr peptide of PN-II yielded a sequence that overlapped with the N-terminal sequence previously reported³ (Fig. 1). Additional sequence was obtained from a PN-II peptide generated by endoproteinase Lys-C. We searched the protein-sequence database of the National Biomedical Research Foundation for sequences with identity to the known sequence of PN-II. Identity was found with the deduced sequence for APP^{4,5} (Fig. 1). The only discrepancy was amino-acid residue 27 in PN-II, which we originally reported as a questionable phenylalanine³; there

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is a histidine residue at the corresponding position in the sequence predicted from the cDNA of APP. The observed sequence identity, together with reports that certain forms of APP possess a protease inhibitory domain^{5,11,12} and protease inhibitory activity¹² indicated that PN-II and APP could be the same or very similar proteins.

Cultured human glioblastoma cells and neuroblastoma cells have been shown to secrete APP²⁰ and to express its messenger RNA^{5,12}. APP and its mRNA have also been detected in human brains^{5,11,12,20}. Likewise, the immunoblots of Fig. 2a show that the PN-II specific monoclonal antibody mAbP2-1 recognized a protein in the culture medium of glioblastoma cells (lane 2) and neuroblastoma cells (lane 3) that seems identical to PN-II present in the culture medium of human fibroblasts (lane 1). Also, mAbP2-1 recognized a similar protein in tissue homogenates prepared from the parietal cortex of normal brain (Fig. 2b, lane 1) or Alzheimer's disease brain (Fig. 2b, lane 2). The presence of PN-II in these cell culture media and brain homogenates was also shown by the formation of complexes with ¹²⁵I-labelled EGF-binding protein that co-migrated with complexes formed between PN-II and EGF-binding protein (data not shown).

FIG. 1 Alignment of the amino-acid sequences of PN-II and APP. Identity was found in segments of the first 120 amino acids of the deduced APP sequence^{4,5}. The N-terminus of PN-II starts at position 18 of APP. Underlined PN-II residues at positions 46–72 were derived from a PN-II CNBr peptide. PN-II residues at positions 46–50 shown in bold represent overlap from the N-terminal sequence of native PN-II and the N-terminal sequence of the PN-II CNBr peptide. The arrow at position 44 indicates an uncertain phenylalanine from the original N-terminal sequence of native PN-II (ref. 3) and is the only discrepancy in the alignments. Underlined PN-II residues at positions 107–116 were derived from a PN-II endoproteinase Lys-C peptide. No determination could be made for position 108.

METHODS. Purified PN-II (~2 nmol) was digested overnight with either CNBr or endoproteinase Lys-C (Boehringer). The resulting peptides were subjected to SDS-PAGE as described by Laemmli²⁴. The peptides were electro-eluted from the gels onto Immobilon polyvinylidene difluoride membranes (Millipore) in a Transblot unit (BioRad Laboratories). After transfer, the membranes were stained with Coomassie Brilliant Blue R-250, de-stained and soaked in several changes of distilled water. A CNBr peptide of relative molecular

FIG. 2 Immunoblot analysis of PN-II in cell-culture media and brain-tissue homogenates. a, Monoclonal antibody mAbP2-1 stained identical proteins in PN-II-enriched culture medium from normal human fibroblasts (lane 1), human glioblastoma cells (lane 2) and human neuroblastoma cells (lane 3). b, This same monoclonal antibody stained apparently identical proteins in tissue homogenates from normal (lane 1) and Alzheimer's disease (lane 2) brain.

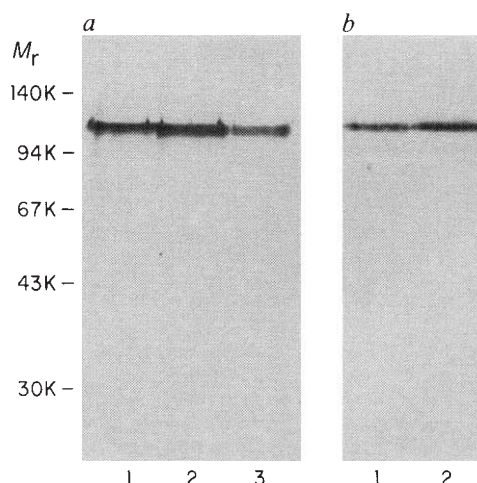
METHODS. The PN-II-specific monoclonal antibody, mAbP2-1, was prepared by fusing P3X63-Ag8.653 myeloma cells with splenocytes from a BALB/c mouse previously immunized with highly purified PN-II. The immunizations, fusion and subcloning were carried out as previously described for the preparation of PN-II-specific monoclonal antibodies²⁶. Enzyme-linked immunosorbent assay (ELISA) demonstrated that mAbP2-1 specifically bound PN-II. The immunoblots show that mAbP2-1 recognized only PN-II in brain-tissue homogenates and cell-culture media. Fibroblasts from normal human neonatal foreskin, human glioblastoma HTB-14 cells and human neuroblastoma HTB-11 cells were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics. Serum-free medium from the cultured cells was concentrated and enriched for PN-II by passing 100 ml of medium through a 1-ml DEAE-Sepharose column; the adsorbed protein was eluted with 2 ml of 1 M NaCl. Human-brain parietal cortex sections were placed in a buffer (20 ml per gram of tissue) containing 200 mM NaCl, 20% glycerol, 1% Triton X-100 and 20 mM potassium phosphate, pH 7.4, homogenized for 5 min using a Polytron homogenizer and centrifuged at 10,000g for 15 min at 4 °C to remove particulates. For immunoblots, samples were subjected to SDS-PAGE; completed gels were soaked in transfer buffer and the proteins were electro-eluted onto Immobilon polyvinylidene difluoride membranes for 2.5 h at 0.4 A in a Transblot unit. The membranes were gently agitated overnight at 25 °C in TBS (50 mM Tris-HCl, 150 mM NaCl,

The antibody mAbP2-1 selectively stained neuritic plaques in tissue sections of Alzheimer's disease brain. Figure 3a shows that mAbP2-1 exhibited intense staining of neuritic plaques in the CA1 region of the hippocampus of a patient with Alzheimer's disease. Staining was not observed in age-matched controls (data not shown). Higher magnification of a neuritic plaque (Fig. 3b) showed that the staining was more pronounced at the periphery.

We tested the ability of purified PN-II to inhibit a variety of serine proteases by using spectrophotometric assays with chromogenic substrates, and found that it was a potent inhibitor of chymotrypsin (Fig. 4a). We have previously shown that PN-II formed SDS-resistant inhibitory complexes with EGF-binding protein, the γ -subunit of nerve growth factor and trypsin¹⁻³. (Note that incubation with excess trypsin resulted in proteolysis of PN-II.) The formation of complexes between chymotrypsin and PN-II was not observed by SDS-PAGE. Stable binding of chymotrypsin to PN-II, however, was identified with a ¹²⁵I-labelled chymotrypsin blotting assay. The autoradiograph shown in Fig. 4b demonstrates that ¹²⁵I-labelled chymotrypsin bound to purified PN-II that had been transferred to a nitrocellulose membrane after SDS-PAGE. This assay showed that

		10	20	30	40
APP	M L P G L A L L L L A A W T A R A L E V P T D G N A G L L A E P Q I A M F C G R				
PN-II			L E V P T D G N A G L L A E P Q I A M F C G R		
		50	60	70	80
APP	L N M H M N V Q N G K W D S D P S G T K T C I D T K E G I L Q Y C Q R V Y P E L				
PN-II	I N M F M N V Q N G K W D S D P S G T K T C I D T K E G I L Q Y				
		90	100	110	120
APP	Q I T N V V E A N Q P V T I Q N W C K R G R K Q C K T H P H F V I P Y K C L V G				
PN-II				T X P H E V I P Y R	

mass 12,000 (*M*_r 12 K) and an endoproteinase Lys-C peptide of *M*_r 20 K were excised from the membranes and directly subjected to N-terminal amino-acid sequence analysis using a gas-phase sequencer (Applied Biosystems, model 475-A) with an on-line microbore phenylthiohydantoin-amino-acid separator and data analyser (Applied Biosystems, models 120-A and 900-A). The N-terminal sequence of PN-II (ref. 3) and two PN-II peptide sequences were searched in the National Biomedical Research Foundation database using the Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin) running on a VAX-VMS computer²⁵.



pH 7.5) containing 0.25% gelatin to block unoccupied sites, and then incubated with mouse monoclonal mAbP2-1 hybridoma-culture supernatant for 1 h at 37 °C with gentle agitation. After several washes with TBS containing 0.05% Tween-20, bound mouse mAbP2-1 was detected with a biotinylated sheep anti-mouse IgG (Amersham) and a streptavidin-horseradish peroxidase complex with several washes between each step. To develop the immunoblots, 48 mg of 4-chloronaphthol were dissolved in 16 ml ice-cold methanol, and this was added to 80 ml ice-cold TBS and then to 64 μ l H₂O₂.

^{125}I -labelled chymotrypsin bound to PN-II after SDS-PAGE of brain-tissue homogenates and cell-culture media from fibroblasts, neuroblastoma cells and glioblastoma cells (Fig. 4c). To further examine the inhibition of chymotrypsin by PN-II, the inhibition constant K_i was measured for the reaction. These studies showed that PN-II was a potent and reversible inhibitor of chymotrypsin with a K_i of 6×10^{-10} M. Reversible inhibition is characteristic of the Kunitz-type serine protease inhibitors²¹, which are homologous to a domain in certain forms of APP^{5,11,12}. Note that incubation of PN-II with chymotrypsin also resulted in proteolytically processed forms of the inhibitor which still retained their ability to bind and inhibit chymotrypsin (data not shown). Similar results have been reported for another Kunitz-type inhibitor, plasma inter- α -trypsin inhibitor²².

Several findings have implicated the involvement of a chymotrypsin-like protease in the deposition of amyloid β -protein in Alzheimer's disease neuritic plaques. A methionine residue flanks the N-terminal side of the peptide bond that is cleaved on release of the amyloid β -protein from APP^{4,5}. This site may be susceptible to cleavage by a chymotrypsin-like protease. It is noteworthy that the serpin α_1 -antichymotrypsin is a component of the amyloid deposits in Alzheimer's disease and its expression is enhanced in Alzheimer's disease brain¹⁰. Here we have presented evidence indicating that the protease inhibitor PN-II is APP and that it effectively inactivates chymotrypsin.

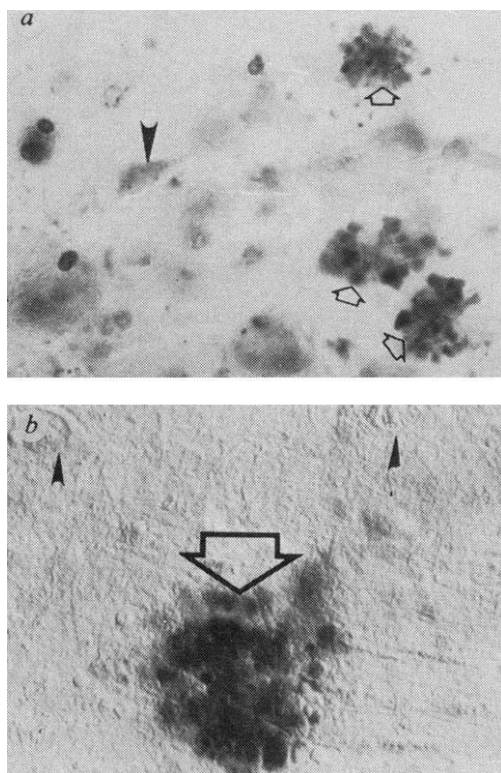


FIG. 3 Immunostaining of Alzheimer's disease neuritic plaques with monoclonal antibody mAbP2-1. *a*, Photomicrograph showing strong immunoperoxidase reaction for PN-II in scattered neuritic plaques (open arrows) in the CA1 region of the hippocampus of a 62-year-old female patient with Alzheimer's disease. Solid arrow points to a pyramidal neuron. Vibratome section (40 μm) viewed by brightfield microscopy ($\times 200$ magnification). *b*, Photomicrograph of a neuritic plaque on higher magnification showing strong immunoperoxidase reaction for PN-II (open arrow) in the CA1 region of the hippocampus. Solid arrow points to pyramidal neurons. Vibratome section (40 μm) viewed by Nomarsky interference microscopy ($\times 1,000$ magnification).

METHODS. Tissues were fixed overnight in 2% paraformaldehyde and 0.01% glutaraldehyde. After washing in PBS, vibratome sections were prepared and subsequently processed using an avidin-biotin complex, immunoperoxidase detection system for PN-II.

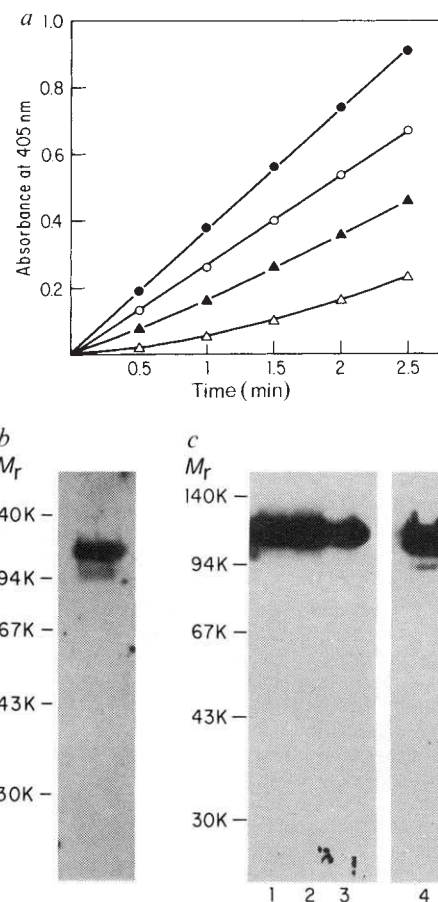


FIG. 4 Inhibition and binding of chymotrypsin by PN-II. *a*, Inhibition of chymotrypsin by PN-II. The molar ratios of PN-II to chymotrypsin were 0:1 ($\bullet$); 0.5:1 ($\circ$); 1:1 ($\blacktriangle$); and 2:1 ($\triangle$). *b*, Binding of ^{125}I -labelled chymotrypsin to purified PN-II after subjecting the PN-II to SDS-PAGE and transferring to a nitrocellulose membrane. *c*, Binding of ^{125}I -labelled chymotrypsin to PN-II in medium of cultures of normal human neonatal foreskin fibroblasts (lane 1), human glioblastoma cells (lane 2), human neuroblastoma cells (lane 3), or in human brain-tissue homogenate (lane 4).

METHODS. K_i was determined by the method of Henderson²⁷ as simplified by Bieth²⁸ and recently described by Pratt and Pizzo²². In brief, increasing amounts of titrated PN-II were incubated with 20 nM active-site titrated chymotrypsin in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 containing 0.1% bovine serum albumin. Incubations were at 37 $^{\circ}\text{C}$ for 0.5–5 min. Remaining protease activity was measured by the addition of buffer containing the chymotrypsin-specific chromogenic substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma). Remaining chymotrypsin activity was monitored at an absorbance of 405 nm. Graphical analysis yielded an apparent K_i , $K_{i,app}$, using the equation $[I]/(1-a) = K_{i,app}(1/a) + [P]$, where $[I]$ and $[P]$ are the initial concentrations of inhibitor and protease, respectively, and a is the remaining fractional protease activity. Because the inhibition is reversible, a correction must be made for the Michaelis constant K_m of the protease-substrate reaction. The K_m for chymotrypsin and substrate was determined independently, and the true K_i was calculated using the equation

$$K_i = \frac{K_{i,app}}{1 + [S]/K_m}$$

Purified PN-II (2 μg) (*b*) or cell culture media or brain homogenates in which the PN-II had been partially purified and concentrated as described in Fig. 2 (*c*), were subjected to SDS-PAGE according to Laemmli²⁴. The completed gel was soaked for 10 min in a transfer buffer consisting of 10 mM sodium bicarbonate, 3 mM sodium carbonate, 20% methanol, pH 9.9, and the PN-II was electro-eluted onto a nitrocellulose membrane for 2 h at 400 mA in Transblot unit (BioRad). After transfer, the nitrocellulose membrane was gently agitated overnight in a solution of TBS containing 0.25% gelatin to block unoccupied sites on the membrane, and then incubated with a solution of TBS containing 20 ng ml^{-1} of ^{125}I -labelled chymotrypsin (specific activity, 5.5×10^5 c.p.m. p.mol^{-1}) for 20 min at 25 $^{\circ}\text{C}$ and then washed twice, each for 10 min, with TBS containing 0.05% Tween-20 and finally washed in TBS. Membranes were dried and exposed to X-ray film for 12–24 h at -70°C .

There is a report that Alzheimer's disease brain contains higher levels of mRNA for APP lacking the domain of the Kunitz-type protease inhibitor than does normal brain²³. Together, these findings indicate that a physiological function for PN-II/APP is the regulation of a chymotrypsin-like protease. This protease could generate amyloid β -protein and possibly other peptides that contribute to the formation of neuritic plaques. Future studies will determine if neural cells bind, internalize and degrade PN-II-protease complexes as do fibroblasts¹⁻³. Alterations in this process could lead to extracellular accumulation of these complexes or of fragments of them. □

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Capping of surface receptors and concomitant cortical tension are generated by conventional myosin

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WE have investigated the role of cytoskeletal contraction in the capping of surface proteins crosslinked by concanavalin A on mutant *Dictyostelium* cells lacking conventional myosin¹. Measurements of cellular deformability to indicate the development of cortical tension² show that cells of the wild-type parental strain, AX4, stiffen early during capping and relax back towards the softer resting state as the process is completed. Mutant cells lacking myosin (*mhcA*⁻) have a lower resting-state stiffness, and fail to stiffen and to cap crosslinked proteins on binding concanavalin A. Hence conventional myosin is essential both for capping and for the concomitant increase in cell stiffness. Furthermore, depletion of cellular ATP by azide causes a 'rigor' contraction in AX4 cells which makes them stiffen and become spherical. By contrast, the *mhcA*⁻ cells fail to respond in these ways. These measurements of cortical tension in non-muscle cells can thus be

TABLE 1 Measurements of cellular contractile stiffening

Cell	Condition	Stiffness (mdyne μm^{-1} $\pm$ s.d.(N))
AX4	Resting	0.41 $\pm$ 0.15 (42)
AX4	Con A (during capping)*	0.91 $\pm$ 0.36 (34)
AX4	Con A (capping completed)*	0.52 $\pm$ 0.25 (15)
<i>mhcA</i> ⁻	Resting	0.28 $\pm$ 0.10 (40)
<i>mhcA</i> ⁻	Con A†	0.35 $\pm$ 0.12 (30)
AX4	Azide‡	2.00 $\pm$ 0.60 (15)
<i>mhcA</i> ⁻	Azide‡	0.46 $\pm$ 0.18 (17)

N is the number of cells measured.

* Stiffness was determined during the first 15 min after con A addition (during capping), or after 30 min incubation with con A (capping completed).

† Cells were measured at times up to 45 min after con A addition.

‡ After 30 min incubation with sodium azide.

Cellular stiffness was measured as described for lymphocytes². *Dictyostelium* cells, grown and prepared as described in the legend to Fig. 1, were allowed to adhere to a glass coverslip for 1-2 min and then immersed in a 10 ml thermostated chamber mounted on the microscope stage. Measurements were made at 22 °C in oxygenated MES buffer, or in MES buffer containing 30 $\mu\text{g ml}^{-1}$ con A or 10 mM sodium azide. The cells were slightly indented to a depth of 0.5-0.8 μm by a glass stylus of 1.5 μm diameter, which moved at a speed of 2.6 $\mu\text{m s}^{-1}$. Stiffness was defined as the resistance force per unit indentation depth, in units of mdyne μm^{-1} . The ingoing curves recorded with the cell pocker were linear, so the stiffness parameter is well defined. The standard deviation represents the variability of stiffness values in the cell population. The precision in measurements of one cell is ± 0.02 mdyne μm^{-1} .

directly correlated with the presence of conventional myosin, demonstrating that contractile tension generated by myosin can drive both a change of cell shape and the capping of crosslinked surface receptors.

The role of the cytoskeleton, and specifically of myosin-dependent contractile forces, in the active redistribution of cell-membrane proteins has been a subject of debate³⁻⁵. Capping of membrane proteins that have been crosslinked by the tetraivalent lectin concanavalin A (con A) has been studied extensively in lymphocytes⁶⁻⁸ and *Dictyostelium*^{9,10}. Not only do actin, myosin and other cytoskeletal proteins co-cap with the con A receptors^{6,7,9} in both types of cell, but the binding of con A to *Dictyostelium* causes a 20-fold increase in the concentration of myosin and a 3-fold increase in actin associated with the Triton-insoluble cytoskeleton⁹. Myosin thick filaments have also been visualized in the *Dictyostelium* cell cortex¹¹. Both immunofluorescence^{6,7,9,11} and mechanical studies² indicate, but do not prove, that a myosin-dependent cytoskeletal contraction drives capping. A more direct test is provided by eliminating myosin, the cytoskeletal protein that generates contractile force. This has now been accomplished in *Dictyostelium* by eliminating through homologous recombination the single gene coding for the heavy chain of conventional myosin¹. The myosin null mutants (*mhcA*⁻) in suspension are defective in cytokinesis, but on a substratum can fragment by a traction-mediated cytoplasmic fission (J.A.S. and S. J. Kron, unpublished results) and reach normal cell size¹. The *mhcA*⁻ cells may have subtle abnormalities in their motion and chemotaxis, similar to those characterized^{12,13} in *Dictyostelium hmm* cells¹², which express only the head fragment of myosin. To determine whether a myosin-dependent contractile process is responsible for cellular stiffening and capping of con A receptors in *Dictyostelium*, we have directly measured changes in these parameters in AX4 wild-type *Dictyostelium* cells and in *mhcA*⁻ myosin null mutants.

The response of AX4 cells to con A is similar to that of lymphocytes². About 50% of the AX4 cells cap fluorescein-labelled con A receptors, with maximal capping occurring 7-10 minutes after addition of con A at 22 °C (Fig. 1a). By contrast, the *mhcA*⁻ myosin null cells fail to cap con A-labelled surface proteins (Fig. 1b). No capping is detected in these cells even after 90 minutes of incubation with con A (data not shown).

FIG. 1 Redistribution of crosslinked con A receptors into a cap depends on the presence of myosin in the cells. Cells were labelled with fluorescein isothiocyanate-labelled con A (FITC-conA). *a*, Time-dependence of con A-capping in AX4 *Dictyostelium* cells. Maximal capping occurred about 10 min after addition of con A and ~50% of the cells capped. At later times most of the ligand was internalized. *b*, Mutant *mhcA*⁻ cells that lack myosin fail to redistribute con A receptors into a cap. The cross-linked surface receptors can cluster and accumulate in contact regions between cells, indicating that the con A receptors are mobile in the membrane of the mutant cells. These cells internalize some of the labelled con A. Scale bar, 10 μ m.

METHODS. The axenic strain of *Dictyostelium*, AX4, and *mhcA*⁻/A5 *Dictyostelium* myosin null mutant cells were grown in tissue culture plates (Falcon) in HL5 defined medium. The medium for *mhcA*⁻ cells contained 6 μ g ml⁻¹ G-418 (ref. 1). The cells were removed by flushing the medium gently with a pipette and then washed in oxygenated MES buffer (20 mM [2(N-morpholino)ethanesulphonic acid], 2 mM MgSO₄, 0.2 mM CaCl₂, pH 6.8; oxygen was bubbled through the buffer for 20 min). Cells were collected by centrifugation at 2,000 r.p.m. for 2 min. For fluorescence microscopy, cells were treated with 30 μ g ml⁻¹ FITC-Con A (Sigma) and swirled in an open beaker. Two minutes before fixation with paraformaldehyde the cells were allowed to adhere to glass coverslips pretreated with

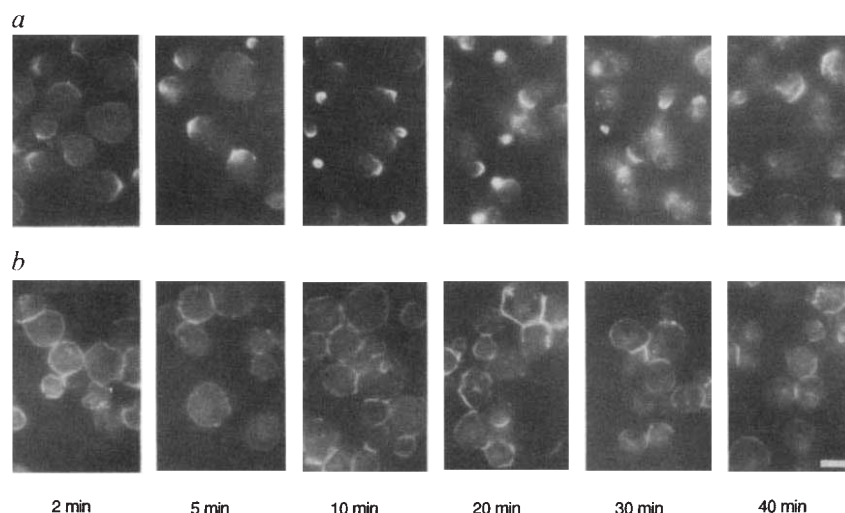
Some internalization of con A-labelled proteins seems to occur in *mhcA*⁻ cells. In experiments complementary to those we describe here (Y. Fukui, A. De Lozanne and J.A.S., submitted), *hmm* cells fail to cap con A, indicating that the tail portion of the myosin, which is involved in thick-filament formation, is essential for the capping process.

Using a direct method to measure cortical tension, we find that AX4 cells stiffen in response to con A, as previously shown for lymphocytes². An increased stiffness of AX4 cells was detectable, starting about two minutes after addition of 30 μ g ml⁻¹ con A. The stiffness increased to 0.90 mdyne μ m⁻¹, which is substantially greater than the 0.41 mdyne μ m⁻¹ value in the resting condition. Following capping, from 30–45 minutes after addition of con A, the average stiffness decreased to 0.52 mdyne μ m⁻¹ (Table 1). As shown previously for lymphocytes², the time-dependence of the mechanical response correlates with the capping of surface receptors.

The myosin null cells have a lower resting stiffness, 0.28 mdyne μ m⁻¹ (Table 1), indicating that some of the stiffness of the resting cells results from myosin-dependent cortical tension. Also, in contrast to the AX4 wild-type cells, the stiffness of the mutant cells increases only slightly from 0.28 mdyne μ m⁻¹ to 0.35 mdyne μ m⁻¹ in response to con A (Table 1) and is not time-dependent. The failure of the mutant cells either to stiffen substantially or to cap indicates that a myosin-dependent contraction is involved in both processes.

These results are consistent with a model for capping in which crosslinking cell-surface proteins by con A activates a myosin-dependent cytoskeletal contraction. This contraction is possibly mediated by the same intracellular responses as in lymphocytes: activation of the phosphoinositide cascade¹⁴, an increase in intracellular calcium¹⁵, and phosphorylation of myosin light chains¹⁶. The resulting isometric tension in the cell cortex causes the increased stiffness. Release of the tension at one location in the cortex allows actin, myosin, other actin-associated proteins and the crosslinked surface proteins to be drawn, possibly by an isotonic contraction, to the opposite pole of the cell to form the cap. Our results demonstrate that *Dictyostelium* cells that lack conventional myosin cannot perform these functions.

To probe further the role of myosin in controlling cellular morphology and also to test the possibility of developing a 'rigor' contraction in non-muscle cells, we treated *Dictyostelium* cells with 10 mM NaN₃ for 30 minutes to deplete cellular ATP. This concentration and incubation period has been shown to



0.1 mg ml⁻¹ poly-L-lysine (Sigma) for 15 min and washed in MES buffer. Cells were fixed in 2% paraformaldehyde in MES buffer for 15 min at the times indicated. Coverslips were washed with MES buffer, inverted onto glass slides in the presence of 50% glycerol, 50% MES and then sealed using clear nail polish. Cells were visualized and photographed using a Zeiss M-35 microscope with a 100 $\times$ oil immersion objective and attached camera.

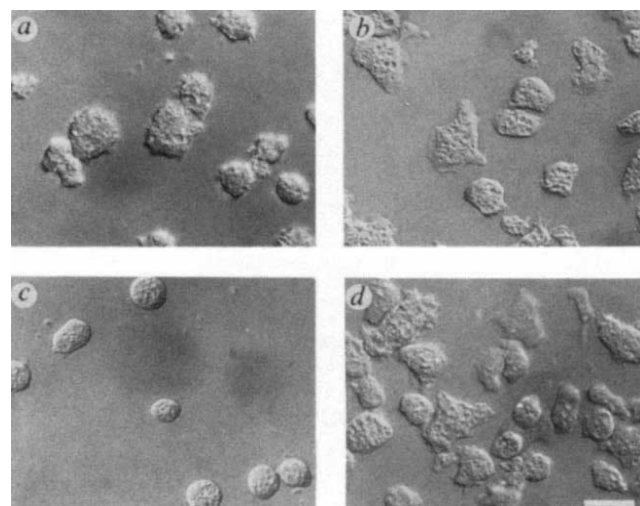


FIG. 2 Azide causes a 'rigor' contraction in *Dictyostelium* cells that have conventional myosin, but does not affect the morphology of myosin null mutants. *a*, *Dictyostelium* AX4 cells in MES buffer; *b*, *mhcA*⁻ *Dictyostelium* cells in MES buffer. These cells tend to spread more and internal vacuoles are more visible. *c*, AX4 cells after 30 min incubation with 10 mM sodium azide. The cells round up and contract and the surface becomes smooth. They tend to detach from the coverslip. *d*, Morphology of *mhcA*⁻ cells and their adhesive properties are not affected by incubation with azide. Scale bar, 20 μ m.

METHODS. AX4 and *mhcA*⁻ cells in MES oxygenated buffer were allowed to adhere to round glass coverslips for 5 min and then inverted onto glass slides holding a drop of MES buffer or MES buffer with 10 mM NaN₃. After 15 min the coverslips were sealed using warm wax and a thin circle of filter paper around the circumference of the coverslip. Cells were visualized using a Zeiss IM-35 microscope with a 40 $\times$ objective, and photographed after 30 min incubation with MES buffer or MES buffer containing 10 mM azide.

reduce the cellular ATP concentration to ~5% of its original level in hepatocytes¹⁷. The AX4 cells respond to azide by immediately retracting spread regions to assume a smooth-surfaced spherical shape (compare Fig. 2a and c) and their stiffness increases 5-fold from 0.41 mdyne μ m⁻¹ to 2.0 mdyne μ m⁻¹ (Table 1). In contrast, the *mhcA*⁻ cells show no detectable morphological response to azide (Fig. 2b and d) and a relatively small increase in stiffness from 0.28 to

0.46 mdyn μm^{-1} (Table 1). This small change could result from osmotic effects caused by inhibition of ion pumps, or from other types of myosin¹⁸, or possibly from other ATP-dependent systems. The effect of azide on the wild-type AX4 cells indicates that, as in skeletal muscle¹⁹, ATP depletion can cause a rigor contraction in *Dictyostelium*. Azide has also been shown to prevent capping of crosslinked surface receptors^{6,10}, presumably by inducing a rigor contraction which fixes the cells in a non-motile state.

Determination of the cellular functions of cytoskeletal proteins has proved difficult because, unlike enzymes, which have readily measurable catalytic activity, cytoskeletal proteins have structural and mechanical functions that are more difficult to measure. Elimination of a specific protein from a cell, either functionally²⁰⁻²² or genetically^{1,23}, has been used to assess qualitative effects on cell morphology or motility, but direct quantitative assay of mechanical function, such as we report for conventional myosin in single non-muscle cells, is a more discriminating test. Although conventional myosin is not required for morphological changes such as lamellar extension during locomotion¹³, it clearly can influence cell shape (Fig. 2), as well as drive a contractile process that moves surface receptors into a cap. Our measurements have demonstrated that capping in *Dictyostelium* is associated with a cellular stiffening and that both capping and stiffening fail to occur in cells lacking conventional myosin. These results support the hypothesis^{2,3,7,9} that stiffening results from an actin-myosin contraction and that this contractile force is responsible for capping. For example, release of tension at

one location would allow crosslinked membrane proteins and associated cytoskeletal components to be drawn together to form a cap at the opposite pole of the cell. Because of similarities in capping in *Dictyostelium* and lymphocytes, it is likely that this mechanism also operates in capping of lymphocyte surface receptors. □

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Complete replacement of mitochondrial DNA in *Drosophila*

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THE introduction of foreign mitochondria or mitochondrial DNA into a cell is a useful technique for clarifying the molecular mechanisms responsible for the maintenance of mitochondria. Novel combinations of mitochondrial and nuclear genomes have been studied in mammalian cells in culture¹⁻⁶ and in yeast^{7,8,14,15}. In *Drosophila*, we have recently constructed heteroplasmic flies possessing both endogenous mitochondrial DNA and foreign mitochondrial DNA by intra- and interspecific transplantation of germ plasm⁹. During the maintenance of these heteroplasmic lines, flies of *D. melanogaster* are produced that no longer possess their

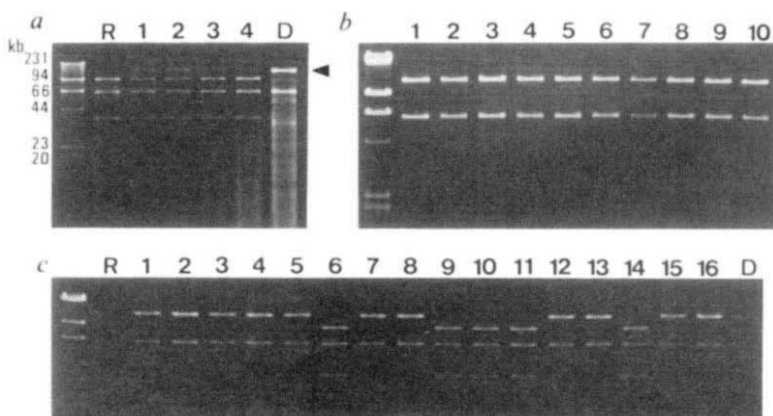
own mitochondrial DNA but retain the foreign mitochondrial DNA from *D. mauritiana*. These flies are fertile and the foreign mitochondrial DNA is stably maintained in their offspring. Here we report the complete replacement of endogenous mitochondrial DNA with that from another multicellular species. Molecular and genetic analysis of this replacement in *Drosophila* should provide new insight into the functional interaction between nuclear and organelle genomes.

D. melanogaster and *D. mauritiana* belong to the *melanogaster* species subgroup in *Drosophila*. An interspecific combination of nuclear and mitochondrial genomes cannot be established by crossing, owing to the sterility of the interspecific hybrids¹⁰. Germ plasm rich in mitochondrial DNA¹¹ was transplanted into freshly laid eggs⁹ using *bw;e¹¹* (brown eye and ebony body colour) of *D. melanogaster* as a recipient, and *D. mauritiana* (g20 strain) as a germ-plasm donor. Mitochondrial DNAs (mtDNAs) of *bw;e¹¹* and g20 can be readily distinguished by their *Hae*III restriction enzyme digest patterns (Fig. 1a).

Four heteroplasmic lines (C5, D2, D3 and F3), established from individual heteroplasmic females⁹ and possessing both recipient (*bw;e¹¹*) and donor(g20)-derived mtDNA (Fig. 1a), were maintained at 25 °C. The proportion of donor mtDNA in progeny flies was monitored in subsequent generations. During

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FIG. 1 Examples of *Hae*III restriction patterns of mtDNA from heteroplasmic lines and sublines. Mitochondrial DNA was extracted from adult flies, digested and separated on 0.8% agarose gels^{9,12}. In the first lane of each gel, fragments from lambda phage DNA digested with *Hind*III are shown as size markers; *bw;e¹¹* (recipient, R) mtDNA and g20 (donor, D) mtDNA are run next to the size marker lane and in the last lane, respectively, in a and c. a, Mitochondrial DNA from the four heteroplasmic lines: C5 (lane 1), D2 (lane 2), D3 (lane 3) and F3 (lane 4), at the second generation. The diagnostic band for g20 mtDNA is indicated by an arrowhead. b, Mitochondrial DNA from sublines established from the D2 line at the 15th generation (lanes 1-10). c, Mitochondrial DNA from sublines established from the C5 (lanes 1-7) and F3 (lanes 8-16) lines at the 32nd generation.



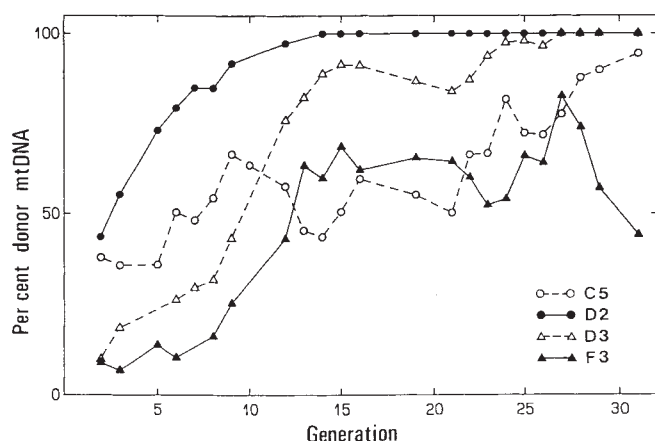
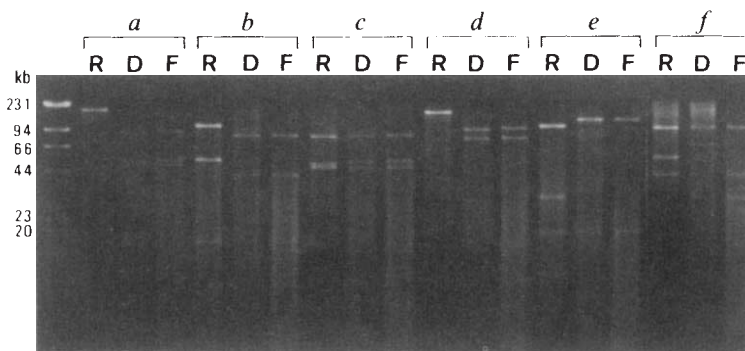


FIG. 2 Change in the proportion of g20 mtDNA in four heteroplasmic lines. These lines were maintained at 25 °C by brother-sister mating of 50–100 flies in each generation. Mitochondrial DNA was extracted from flies used as parents for the next generation and the proportion of g20 mtDNA was determined⁹.

FIG. 3 Restriction patterns of *bw*; *e*¹¹ (recipient, R), g20 (donor, D) and D2 (fixed, F) mtDNA. These patterns are consistent with the restriction maps of mtDNA reported by Solignac *et al.*¹³. a, *Bgl*II; b, *Eco*RI; c, *Hind*III; d, *Hpa*I; e, *Pvu*II; f, *Sac*I.



the first ten generations, the proportion of g20 mtDNA gradually increased in all four lines (Fig. 2). Finally, *bw*; *e*¹¹ mtDNA ceased to be detectable in the D2 and D3 lines after 14 and 27 generations, respectively. To date, we find that recipient mtDNA has been absent for more than 25 generations. The interspecific combination of nuclear and mitochondrial genomes apparently does not hinder the survival of a host fly, at least under these conditions.

To determine whether recipient mitochondria are lost in each female, ten sublines were established from individual females inseminated by their brothers in the D2 line at the 15th generation, and the progeny mtDNA was digested with *Hae*III. All ten sublines showed the restriction pattern of g20 mtDNA and none showed the mtDNA band characteristic of *bw*; *e*¹¹ (Fig. 1b). We conclude that the mtDNA of *bw*; *e*¹¹ (*D. melanogaster*) has been completely replaced by mtDNA of g20 (*D. mauritiana*) in each female in the D2 line.

Replacement of *bw*; *e*¹¹ mtDNA and fixation of g20 mtDNA was confirmed using another six restriction enzymes: *Bgl*II, *Eco*RI, *Hind*III, *Hpa*I, *Pvu*II and *Sac*I. These enzymes gave restriction patterns for mtDNA of the D2 line that were the same as those from the original g20 strain, but differed from those derived from *bw*; *e*¹¹ mtDNA (Fig. 3). We further verified fixation of g20 mtDNA by Southern hybridization, using a ³²P-labelled mtDNA fragment of *D. melanogaster* as a probe: this probe hybridized to each of the bands diagnostic for *bw*; *e*¹¹ and g20 mtDNA (data not shown). It is therefore evident that mtDNA of the D2 line originates from the donor (g20) mitochondria and is not derived from a change in *bw*; *e*¹¹ mtDNA by mutation, recombination or fusion with g20 mtDNA.

In the C5 and F3 lines, two types of mtDNA co-exist among the progeny even at the 32nd generation. To determine whether this results from a heteroplasmic state in individual flies or from the co-existence of flies each carrying one type of mtDNA, we

examined the *Hae*III restriction pattern of mtDNA from the progeny of the 14 and 16 sublines of C5 and F3, respectively. All these sublines were found to carry only one type of mtDNA: either *bw*; *e*¹¹ in 2/14 of C5 and in 7/16 of F3, or g20 in 12/14 of C5 and in 9/16 of F3 (Fig. 1c). Fixation of foreign mtDNA in each female therefore also occurs frequently in these two lines.

D. mauritiana mtDNA can be retained in the absence of its own nuclear genome, although in cultured mammalian cells, mitochondria may depend on the presence of species-specific nuclear gene products for their own survival^{1–3}. We marked the second and third chromosomes of *D. melanogaster* with recessive genetic markers of *bw* and *e*¹¹, respectively. All flies emerged with the *bw*; *e*¹¹ phenotype and so there could have been no contamination by *D. mauritiana* nuclei during the transplantation of germ plasma.

Fixation from the heteroplasmic state was significantly biased towards a preference for donor mtDNA among the individuals in the four lines. Although the reason for this is not yet clear, *D. mauritiana* mtDNA is apparently distinguishable from *D. melanogaster* mtDNA, in spite of the extensive homology

between their mitochondrial genomes¹². In cultured somatic cells, cells with only one type of mtDNA have been selected from heteroplasmic cells using medium containing chloramphenicol, for example^{2–6}. It is significant that we have found preferential fixation of foreign mtDNA in the absence of selective conditions.

It remains to clarify the mechanisms by which the propagation of *D. mauritiana* mtDNA and of chimaeric mitochondria encoded by both *D. mauritiana* mtDNA and *D. melanogaster* nuclear DNA, are regulated under the nuclear genome of *D. melanogaster*. Experiments are in progress on reciprocal combination and on other intra- or interspecific combinations between nuclear and mitochondrial genomes, which aim to clarify the factors involved in regulating the maintenance of mtDNA and mitochondria. □

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